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Effects of Developmental Exposures of Two Emerging Environmental Toxicants on Estrogen-Sensitive Endpoints

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A Thesis Presented

By

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ABSTRACT

EFFECTS OF DEVELOPMENTAL EXPOSURES OF TWO EMERGING ENVIRONMENTAL TOXICANTS ON ESTROGEN-SENSITIVE ENDPOINTS

MAY 2015

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Background: Humans have created thousands of synthetic chemicals that are released into the environment, causing widespread exposures of wildlife and humans alike. Some of these chemicals are known to disrupt one or more aspects of hormone action, with a subset inducing abnormalities in endocrine related tissues and organs such as the reproductive system. Bisphenol S (BPS) and tetrabromobisphenol A (TBBPA) are two chemicals commonly used in consumer products and are suspected to have endocrine disrupting properties based on their similar chemical structures to known endocrine disrupting chemicals (EDCs).

Objectives: To determine whether perinatal exposure to BPS or TBBPA induced abnormalities in ovarian and uterine tissue at the organ, tissue, cellular or molecular levels of biological organization.

Methods: Pregnant female mice were administered vehicle, BPS or TBBPA during pregnancy and lactation. From postnatal day (PND) 19-21, two female offspring from each litter were administered either oil or ethinyl estradiol, and killed at PND22. One additional female per litter was killed at week 16. The reproductive tracts were examined

for gross morphological defects and then fixed and frozen for basic histological, immunohistological and gene-expression assessments.

Results: Our study identified significant differences in ovarian follicular formation and gene expression after developmental exposures to BPS or TBBPA. Most effects were observed at PND22, and were apparent after an estrogen challenge; however, these differences were not observed at 16 weeks of age suggesting that changes induced by BPS and TBBPA are age dependent.

Conclusions: Our results suggest that TBBPA and BPS alter development of the female reproductive tracts, with the most obvious effects on the pre-pubertal ovary after developmental BPS treatment. These findings are among the first to examine the effects of these two chemicals and indicate that widespread exposures to BPS and TBBPA may have implications for public health. Further, evidence regarding negative health effects after animal exposures to widely available EDCs may explain the global increase in female reproductive health abnormalities in humans.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Introduction to Endocrine Disrupting Chemicals

Today, both humans and wildlife live in an environment that contains a multitude of man-made chemicals which have been produced for use as pesticides, industrial compounds, in personal care products, fertilizers, plastics and other packaging materials, pharmaceuticals, and others. Unfortunately, these compounds can have unintended health outcomes on biota. Subsets of these chemicals are capable of mimicking or interfering with the actions of hormones and thus cause disturbances within the endocrine systems of exposed humans and animals. When disturbances to hormone signaling processes cause adverse health effects in the organism itself or in its offspring, they are referred to as endocrine disrupting chemicals (EDCs) (Bergman et al. 2013). In 2009, the Endocrine Society described EDCs as "compounds, either natural or synthetic, which through environmental or inappropriate developmental exposures alters the hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment" (Diamanti-Kandarakis et al. 2009). In 2012, the Endocrine Society simplified this definition, describing an EDC as "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action" (Zoeller et al. 2012) The concept of "hormone action" was further described as the ability of an exogenous chemical to activate a hormone receptor either directly or indirectly resulting in physiological effects (Zoeller et al. 2014).

The majority of EDCs that are present in the environment are products of human manufacturing; however, there are also naturally occurring substances that are able to

disrupt endocrine pathways. These natural EDCs include metals such as arsenic, cadmium, lead and mercury; natural hormones such as estradiol and testosterone excreted from animals and humans; and phytoestrogens such as isoflavonoids and coumestans derived from plants (Bergman et al. 2013). Although these naturally occurring EDCs have potential adverse effects that should be accounted for, and in fact there are documented examples of these natural EDCs disrupting reproductive health, anthropogenic EDCs pose threats to human and wildlife health while providing important paths for public health interventions. This report will focus solely on anthropogenic EDCs.

The field dedicated to the study of EDCs is fairly new, and in fact the term 'endocrine disruptor' was first coined in 2001 (Vandenberg et al. 2015). Up until the early 2000s, the majority of knowledge concerning compounds that interfere with hormone signaling was based on studies involving chemicals created for and used in industry, agriculture and pharmaceuticals. These studies focused on a very limited number of environmentally persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and polychlorinated dibenzofurans (PCDFs), which are reviewed in (Bergman et al. 2013). Apprehension within the scientific community toward these anthropogenic compounds and others did not begin to rise until a series of unusual phenomena relating man-made chemicals to the decline of human, plant and wildlife wellbeing started appearing. The earliest of these occurrences involved the use of synthetic chemicals such as diethylstilbestrol (DES) in the 1930s-1970s, PCBs from 1929-1980s and DDT produced from the 1940s through the present time (Li and Macdonald 2005; Marty et al. 2011). Extensive studies surrounding

these chemicals, and the growing field of biomonitoring and exposure science, sparked a realization that the number of EDCs that one may encounter on a daily basis is astronomically greater than what was expected in past years, and that sources of exposures may vary greatly (Needham et al. 2005; Needham et al. 2008; Woodruff et al. 2011). Within the past decade, it has been revealed that EDCs originate from a wide range of sources including additives in consumer goods and materials such as cosmetics and other personal care products, food packaging, cleaning products, plastics and pesticides, among others. For years, this wide range of commercially available chemicals was excluded from the scope of scientific and health related concern because of expectations that these chemicals were well tested for toxicity. Currently, although the criteria for characterizing compounds as EDCs differs somewhat by different groups, more than 1000 compounds are listed as putative EDCs in the US FDA's Endocrine Disruptor Knowledgebase and on lists maintained by groups such as the Endocrine Disruptor Exchange (Vandenberg et al. 2013b).

The incidence of chronic diseases such as certain cancers, diabetes, obesity, and cardiovascular disease (among many others) are increasing at alarming rates and are collectively the leading causes of illness and death globally, surpassing communicable disease rates even in developing countries (Barouki et al. 2012; WHO 2011). According to the WHO, the expected number of annual cancer cases worldwide will increase from 14 million in 2012 to 22 million within the next 2 decades. In many instances, factors such as genetics, nutrition and lifestyle are not able to fully explain these striking increases in chronic disease. Despite this lack of etiological explanations, research conducted in wildlife and laboratory animal populations point to environmental factors

such as chemical exposures as likely contributors to rising incidences of chronic diseases. It is important to recognize the burden that these diseases place on societies worldwide. The increasing rates of chronic disease pose concerns for public health agencies as they drive the need for increased medical attention and services. Costs that accompany health care required to treat these diseases put drastic stress on economies; for example, it is estimated that global spending on diabetes alone is expected to rise to $490 US billion in 2030, which is 12% of all health care spending (Zhang et al., 2010). Increased spending on health care related to chronic illness and diseases is referred to as the "global burden of disease" and is among the current leading public health concerns globally.

There are a few key factors that designate EDCs as environmental health concerns. First, as stated above, EDCs have the ability to interact with normal hormonal processes and functions within an organism, potentially causing disturbances in major physiological systems such as development and reproduction, resulting in adverse health effects. Second, although not true of every EDC, many of these compounds tend to be lipophilic molecules, and thus are efficiently stored in fat deposits and fluids of the body at a faster rate than they are excreted, a process known as bioaccumulation. In pregnant females, these lipophilic substances can be passed to neonates by crossing the placental barrier, thus exposing the fetus to a variety of concentrations and mixtures of potentially hazardous components (Barr et al. 2007). Lastly, many EDCs and/or their byproducts have the ability to accumulate with increasing order within the food chain, a process known as biomagnification, which allows these chemicals to accumulate in adipose tissue of living organisms and persist long after the chemical was first released into the

environment. This process produces the most risk to organisms at the top of the food chain such as humans and other high-order carnivorous species.

1.2 Bisphenol S and Tetrabromobisphenol A

Bisphenol S (BPS) is a chemical that is structurally similar to Bisphenol A (BPA) (Figure 1), a known EDC that has been shown to induce adverse effects at low doses, especially in reproduction and developmental outcomes (Richter et al. 2007; Rochester 2013; Vandenberg et al. 2010; Vandenberg et al. 2013a). BPA is predominantly used in the production of polycarbonate plastics, both of industrial and consumer grade; thermal papers commonly used for receipt paper; epoxy linings of food and drink containers; as well as other various consumer and industrial products including toys, medical equipment, and sports safety equipment, among others (Geens et al. 2012; Huang et al. 2012). Human exposure to BPA occurs predominantly by two routes of exposure: absorption through the skin or by ingestion; inhalation may also be possible as BPA is found in air and dust samples (Vandenberg et al. 2013a). BPA is an extremely lipophilic molecule allowing it to be absorbed through the skin by mere contact with items containing BPA, such as thermal receipt paper (Biedermann et al. 2010; Zalko et al. 2011). Additionally, BPA leaches out of food and drink packaging and enters the body via ingestion (Vandenberg et al. 2007).

Over the past decade, public knowledge regarding adverse outcomes related to BPA exposure has increased, resulting in consumers' unwillingness to buy products containing this compound. To appease consumer concerns, industries such as the largest thermal receipt paper supplier in the US have begun to replace BPA with other related

compounds such as BPS (Appleton 2010). Currently, knowledge regarding potential adverse health effects induced by BPS exposure is limited. However, due to its analogous structure to BPA, experts suspect that BPS has the potential to induce negative health effects at low doses and hypothesize that BPS has endocrine disrupting abilities.

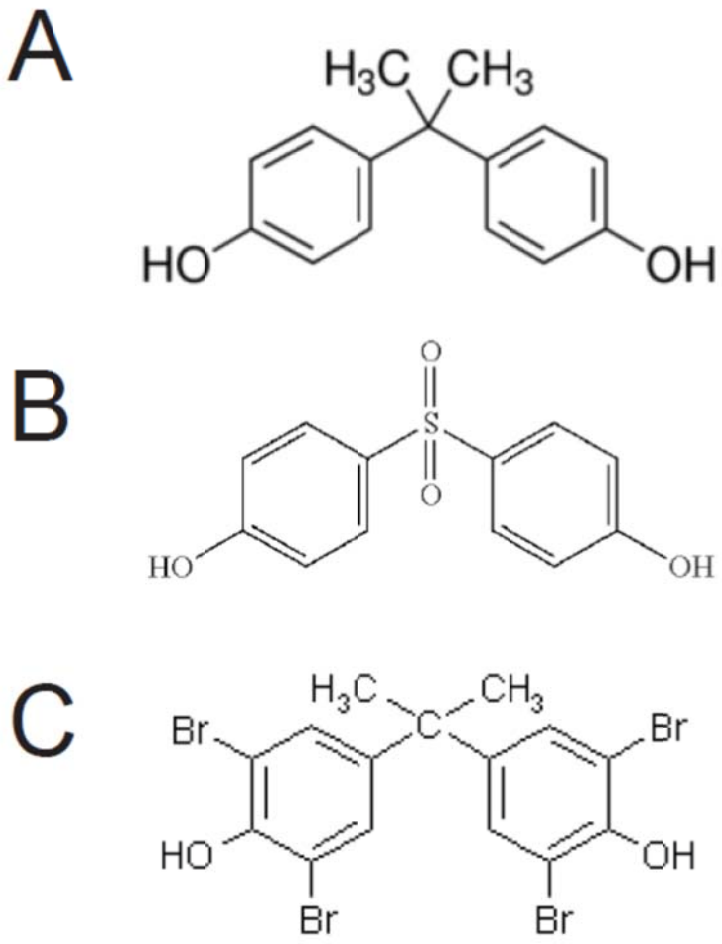


Figure 1. Chemical structures. A) bisphenol A (BPA), B) bisphenol S (BPS), C) tetrabromobisphenol A (TBBPA).

Like BPA, human exposure to BPS occurs predominantly by two routes of exposure, either by absorption through the skin or by ingestion. BPS is lipophilic, allowing it to be absorbed through the skin after contact with items containing the

chemical, such as thermal receipt paper (Porras et al. 2014). BPS also leaches out of food and drink packaging and enters the body via ingestion (Bittner et al. 2014; Ozaki et al. 2004) and is also present in dust, which can ultimately enter the body through ingestion (Liao et al. 2012b).

As discussed elsewhere (Vandenberg et al. 2015), the introduction of BPS to the consumer market as a replacement of BPA may be an example of a regrettable replacement, where one compound is phased out and another with toxicity is used in its place. If BPS does have endocrine disrupting properties, this would be an emerging public health concern as approximately 81% of humans have traceable levels of BPS metabolites in their urine; Japan and the US have the highest BPS detection frequencies which may be attributed to the replacement of BPA with BPS in these two countries (Liao et al. 2012a).

Tetrabromobisphenol A (TBBPA) is also a chemical derivative of BPA created by brominating BPA in an organic solvent (Figure 1). TBBPA is used as a reactive flame retardant that is chemically bound to epoxy and polycarbonate resins used in the manufacturing of electronic circuit boards and textile adhesives, and is used worldwide (Alaee et al. 2003). The purpose of flame retardants is to decrease a product's chance of igniting. Because this compound is released or shed from many consumer products, TBBPA is present in indoor air, dust and river sediment; it enters the body through oral ingestion via dust particles or from contaminated food or water (Butte and Heinzow 2002; Oberg et al. 2002).

There are two main concerns regarding TBBPA and its effects on health. The first concern is that TBBPA, like BPS, is structurally similar to BPA; as structure is often

predictive of biological action, it has been hypothesized that it has the potential to act as an EDC. The second concern is that TBBPA is persistent in the environment due to its bromine groups which allows TBBPA to bioaccumulate within the body including blood and adipose tissue (Jakobsson et al. 2002; Shen et al. 2012; Thomsen et al. 2001). One study observed biomagnification of TBBPA up through wild herring gull food chains and also detected TBBPA in herring gull eggs, consistent with the substance's persistence in the environment (Letcher and Chu 2010). A human study of French mothers found concentrations of TBBPA ranging from 0.062 to 37 ng/g fat in breast milk (Cariou et al. 2008). More recently, TBBPA was found in breast milk of a cohort of Japanese women at concentrations of 0.14 to 8.7ng/g lipid (Nakao et al. 2015). These examples emphasize that exposures to TBBPA can occur in developing young in both wildlife and human populations. Similar to BPS, controlled exposure assessments regarding TBBPA and resulting health outcomes remain largely unstudied.

Despite meager data available that examines adverse health effects induced by BPS and TBBPA, some recent studies suggest that these two compounds have endocrine disrupting properties. Molina-Molina et al. observed that BPS and TBBPA are able to disrupt multiple nuclear receptors *in vitro* and therefore may have the ability to interfere with the endocrine system (Molina-Molina et al. 2013). BPS was found to be an agonist for both estrogen receptor α (ERα) and estrogen receptor β (ERβ) and TBBPA was found to be a weak agonist for human pregnane X receptor (PXR). This study also revealed that BPS but not TBBPA was able to stimulate cell proliferation in MCF-7 cells and inhibit proliferation of MCF-7 AR1 cells. These findings suggest that BPS has estrogenic and androgenic properties and is able to induce estrogen and androgen

dependent outcomes, whereas TBBPA is able to induce xenobiotic detoxification through binding with PXR. However, this study did not observe estrogen regulation effects associated with TBBPA exposure. In contrary, Olsen and colleagues found that TBBPA was able to bind to estrogen receptor isolated from MCF-7 cells, increase levels of estrogen-regulated proteins and induce progesterone receptor (Olsen et al. 2003). Finally, although studies of BPS in mammals are limited, studies in zebrafish support the hypothesis that this chemical can disrupt both reproductive and developmental endpoints (Ji et al. 2013; Kinch et al. 2015; Naderi et al. 2014).

1.2.1 Regulation of BPS and TBBPA

In 1995, the United Nations Environment Programme (UNEP) called for a global recognition and action against further production of persistent chemicals. In 2001, an international environmental treaty known as "The Stockholm Convention" composed guidelines for a select group of chemicals, coined POPs (persistent organic pollutants), which are known to be highly persistent and are capable of long distance transport in the environment. These guidelines set a plan to regulate, reduce and/or completely discontinue the use and production of these compounds. In theory, the Stockholm Convention would apply to TBBPA however; TBBPA is not currently listed as a POP in the Stockholm Convention. In fact, TBBPA is the primary flame retardant used worldwide, accounting for 58.7% of total brominated flame retardant use.

The use of BPS in consumer products is currently increasing due to restrictions put in place by the US Environmental Protection Agency (USEPA), Health Canada, and the European Food Safety Authority (EFSA) that limit the use of BPA in consumer

products (Kinch et al. 2015). Heightened consumer knowledge and concern regarding the use of BPA in consumer products also drives product manufacturers to replace BPA with BPS in order to produce "BPA-free" items, thus ameliorating consumer demand for "safer" alternatives. Currently, no US government agency tests for toxicity of new materials before they are used in consumer products and released to the public (Kinch et al. 2015).

The use of TBBPA and BPS in consumer products is largely unregulated worldwide. Due to the lack of control regarding the use of these chemicals, items containing BPS and TBBPA continue to be available to consumers, leading to widespread human exposures to these two environmental toxicants.

1.3 The issue of risk assessment

The risk assessment process involves four steps: first, hazard identification requires that the biological/toxicological effects of a compound are assessed, typically using standard protocols. Next, dose response assessments are performed, allowing for the prediction of specific hazards at specific doses, as well as the prediction of hazards at doses that were not tested. In the third step, exposure assessments are conducted; this process allows for the identification of sources and routes of exposure. Finally, the data from these first three steps is combined in a risk characterization process, and the risk to the population is estimated.

Risk assessment is often criticized as a non-scientific process, particularly because of studies showing that risk assessment decisions rely on 'expert judgment' and are often not reproducible (Frumkin 2005). Risk assessments for EDCs have also been

challenged, although the issues that influence assessments for this class of compounds differ somewhat from the concerns that have been raised regarding risk assessments in general. These matters are discussed in some depth below.

First, traditional risk assessments operate under the assumption that all chemicals act through a linear dose-response mechanism. This theory encompasses an expectation of increasing response for a set endpoint with increasing concentration of chemical in a linear fashion. Under this assumption, not only are higher doses expected to induce greater effects, there is an assumption that the effects at lower doses can be predicted based on high dose studies. Importantly, this "one model fits all" ideology should be applied to all types of chemicals when assessing associated risks. It has been shown that natural hormones as well as EDCs can defy the expectation of linear or monotonic dose response curves, and instead can include more complicated, non-linear dose responses including non-monotonic dose responses curves (NMDRCs). The term NMDRC simply means that the slope of the dose response curve changes from positive to negative or visa versa at some point of change in dose, usually resulting in a U-shaped or inverted U-shaped dose-response curve. This topic has been reviewed in detail by Vandenberg and colleagues (Vandenberg et al. 2012).

Hormones act via high-affinity binding to specific proteins known as receptors. Downstream effects of hormones are induced when a hormone successfully binds to a receptor and activates a cascade of cellular events including gene transcription. Hormone receptors are limited and present only in specific cells and tissues within an organism. If receptors are not present, hormones will not have the ability to act or induce a response. Considering these hormone-receptor interactions, there are a number of mechanisms that

have been characterized, largely by endocrinologists, to explain how hormones and EDCs can induce NMDRCs. One mechanism is explained by the non-linear relationship between hormone concentration, number of bound receptors, and biological effects (Welshons et al. 2003). Due to limited number of hormone receptors, receptors become saturated when hormone concentrations are high. In this case, response is not solely dependent upon the concentration of hormone but also dependent upon the number of bound receptors as response is dictated by the number of bound hormone-receptor complexes; the greater the number of bound hormone-receptor complexes, the greater the magnitude of response.

Other factors that can contribute to the manifestation of a NMDRC have also been described. The first of these factors is the possibility of receptor down-regulation in the presence of a high concentration of hormone. At high hormone concentration, the rate of receptors lost (due to movement of bound receptors to other sites within the cell such as the nucleus) is greater than the rate of receptor replenishment, resulting in a response-limiting factor. Another factor that contributes to NMDRCs is the ability of some hormones to bind to multiple different types of receptors; this affinity to different receptors is typically based on the concentration of hormone. In this case, at low concentrations the hormone may only bind to receptors that have a very high affinity for that particular hormone whereas at high hormone concentrations the hormone may also bind to receptors that have a lower affinity for that particular hormone; the resulting observed response is due to the hormone binding with multiple classes of receptors.

Endogenous estrogens and xenoestrogens both follow this receptor affinity factor and can produce NMDRCs. An example of this factor is illustrated by the response of

estrogen dependent breast cancer cells that are treated with Tamoxifen, resulting in what is known as “Tamoxifen flare”. At low doses of Tamoxifen, breast cancer cells proliferate, but as concentrations increase in the blood, the “therapeutic range” is achieved, and cancer cells stop proliferating. At higher doses, apoptosis is induced in these cancer cells, and at even higher concentrations, Tamoxifen induces systemic toxicity. In this example, the least toxicity is observed at a moderate dose of the drug (Reddel and Sutherland 1984).

Another related issue that has challenged traditional risk assessment methods is the ability of EDCs to induce adverse effects even when exposures occur at low doses or concentrations. Although the term “low dose” has many different definitions, the National Toxicology Program (NTP) defines low dose effects as “ any biological changes 1) occurring in the range of typical human exposures or 2) occurring at doses lower than those typically used in standard testing protocols” (Melnick et al. 2002). As mentioned above, a biological response can occur only when a hormone successfully binds to its receptor. Low dose effects are possible because receptors are rarely if ever saturated, meaning that even low concentration of hormone or EDC can increase the number of receptors bound, resulting in maximal response even at a low dose.

Finally, the method used to assess the effects of EDCs have been challenged, with some suggesting that the endpoints examined in traditional guideline studies are not relevant to human diseases of concern (Myers et al. 2009a; Myers et al. 2009b). In risk assessments, EDCs are typically treated the same as every other chemical, i.e. assumed to have general toxicity, and assessed based on the linear response model. If chemicals that act as hormone receptor agonists are only tested at doses where a traditional toxic

response (i.e. death, loss of body weight) is expected based on the linear response model, entire responses are left unassessed at lower doses. Unfortunately, this reliance on an expectation of a monotonic dose response may leave entire groups of chemicals such as potential EDCs inadequately assessed.

1.4 The ovary

The ovaries are essential female organs that carry out crucial roles in both the endocrine and reproductive systems. The ovaries house germ cells and also generate and release multiple steroidal and non-steroidal hormones directly into the blood stream; these hormones play important roles in endocrine feedback systems. Mammalian females possess a maximum number of germ cells or oocytes at birth. Their numbers continually decrease after birth until all oocytes have been ovulated or have died via various apoptotic pathways; when all oocytes have been expended, the female enters a period known as menopause. Human females are born with approximately 2 million primordial oocytes; by the time puberty is reached, only about 400,000 remain (Kovacs and Ojeda 2011). The number of primordial oocytes present in mouse ovaries is highly debatable because follicle counting methods involve a great amount of error and numbers vary greatly depending on the strain of animal and methods used (Tilly 2003). The total estimated number of follicles in the mouse ovary reported varies from 3,500 to 30,000 (Pepling and Spradling 2001).

1.4.1 Follicle development

At birth, the oocytes present in the ovary are known as primary oocytes. These primary oocytes undergo stages of follicle maturation based on changes in concentration of gonadotropins, luteinizing hormone (LH) and follicular stimulating hormone (FSH), which are released from the anterior pituitary. Ovulation in humans is regulated by a cyclic mechanism known as the menstrual cycle; one oocyte is ovulated approximately every 29.5 days. Ovulation in the mouse follows a different pattern known as the estrus cycle; environmental cues such as amount of light signal ovulation of multiple oocytes (Gilbert 2010a). The first phase of follicle development is categorized by a series of developmental maturation steps where follicles can be categorized as follows: 1) Primordial follicles include primary oocytes surrounded by a flat layer of epithelial cells. 2) Primary follicles contain the oocyte and a single or double layer of granulosa cells that are formed from the epithelial cells. Granulosa cells have FSH receptors and their main function is to convert androgens to estradiol. 3) Secondary follicles contain the oocyte surrounded by more than two layers of granulosa cells and an outer layer of theca cells that are derived from stromal cells. Theca cells have LH receptors and produce androstenedione, which is used by granulosa cells to convert androgens to estradiol. 4) Antral (or graafian) follicles contain the oocyte surrounded by two or more layers of granulosa cells, outer theca cell layers and contain a large follicular fluid filled compartment known as the antrum (with a high concentration of steroids and local growth factors).

After follicular maturation is complete, the second stage of the follicular phase begins. In this second phase, mature follicles become dominant and each mature follicle expels its ovum into the fallopian tube where it can be fertilized in a process known as

ovulation. After ovulation, the egg-less follicle will recede and reorganize into what is known as the corpus luteum, which contains luteinized granulosa cells that secrete progesterone, the major hormone responsible for the maintenance of pregnancy. In the event that fertilization fails to occur, the luteal cells apoptose and progesterone secretion ceases; a scar-like structure known as the corpus albicans replaces the corpus luteum and the follicular cycle begins again (Kovacs and Ojeda 2011).

Figure 2 shows representative images of the various stages of follicle

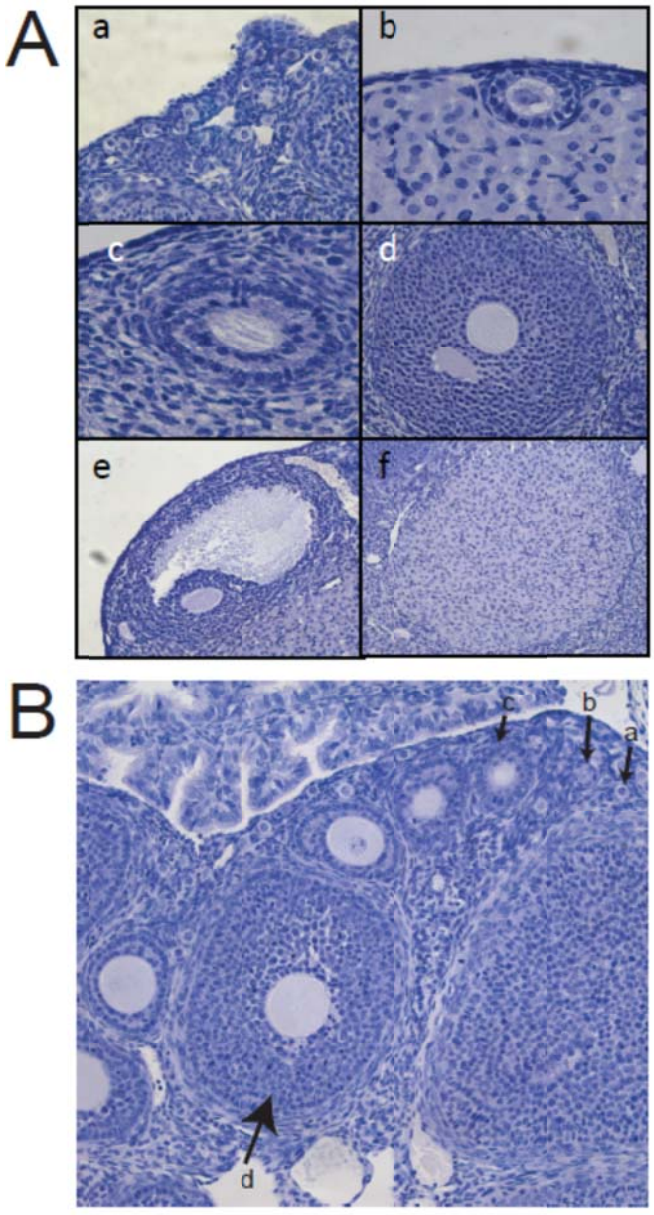


Figure 2. Ovarian follicles by stage. A) Histological images of primordial (a), primary-1 (b), primary-2 (c), secondary (d) and antral follicles (e) as well as a corpus luteum (f). B) Relative position of follicles in a PND22 ovary. Lower case letters are consistent with the follicle stages shown in A.

development in a mouse ovary. An image showing the histological appearance of a corpus luteum is also shown.

1.4.2 Development of the ovary/oogenesis

The ovary and the female germ-line undergo development during prenatal and postnatal development, and are marked by two rounds of meiosis. In mice, primordial germ-cells migrate to the ovaries at approximately days 7-11 of gestation, and germ-cell meiosis I begins at gestational day 13, when germ-cells begin to proliferate and develop into primordial follicles and follicles (Borum 1961; Smith et al. 2014). Primordial germ-cells assemble within the ovary into what is referred to as the "follicle reserve" or "oocyte nest" (Kerr et al. 2013; Uzumcu et al. 2012). In humans, the process of primordial follicle assembly occurs during gestation and is complete by birth whereas in rodents, primordial follicle reserves do not completely form until postnatal day 2-3 (Kerr et al. 2013). These reserves contain a finite number of primordial follicles that will either mature through the stages of follicular development (discussed in Section 1.4.1) and ovulate or will die throughout an individuals' lifespan; about 99.9% of growing follicles are atretic (Uzumcu et al. 2012). Ultimately, the number of viable primordial follicles present at or near birth dictates the length of the reproductive life span. These primordial follicles remain inactive in meiotic arrest of the diplotene stage of prophase I until puberty, when groups of primordial follicles begin follicular growth. At puberty, LH surges from the pituitary will signal select primordial follicles to continue and complete meiosis I. When the follicles mature into a secondary follicle, meiosis II begins but halts at metaphase. Further continuation of meiosis II can only be completed if fertilization occurs. In the

human, some oocytes remain in meiotic prophase arrest for nearly 50 years (Gilbert 2010b).

1.4.3 Ovarian steroid hormones

There are three key families of hormones that are essential for the control and maintenance of ovarian maturation as well as endocrine feedback loops that involve the ovary. These families include estrogens, androgens and progestins. The primary estrogen secreted by the ovary is 17β-estradiol (E_2). In the ovary, E_2 primarily acts via binding to estrogen receptor (ER)-β, which is present in granulosa cells. E_2 induces proliferation and decreases apoptosis of granulosa cells, as well as maintains FSH responsiveness and creates LH receptors. Testosterone is the primary androgen that participates in ovarian feedback loops. Testosterone binds to androgen receptors (AR), which are present in granulosa cells and increases E_2 and progesterone secretion through FSH stimulation. Progesterone is the major hormone in the progestin family. Like AR and ERβ, progesterone receptors (PR) are expressed in granulosa cells. Progesterone, known as the "pregnancy hormone", inhibits E_2 production that is induced by FSH (Kovacs and Ojeda 2011). These three classes of hormones are responsible for follicular development (discussed above) as well as for providing input needed to control other endocrine hormones outside of the ovary.

1.4.4 The hypothalamic-pituitary-ovarian (HPO) axis

Female reproductive hormones are regulated by a system of endocrine organs including the hypothalamus, the anterior pituitary gland, and the ovaries, known as the

hypothalamic-pituitary-ovarian (HPO) axis (Figure 3). The HPO axis is unique in that it is the only endocrine axis system that exerts both a stimulatory (positive) and an inhibitory (negative) feedback loop (Kovacs and Ojeda 2011). These feedback systems are principally controlled by E_2 (as discussed in more detail below); however, other steroid hormones and proteins contribute to overall HPO functions as well.

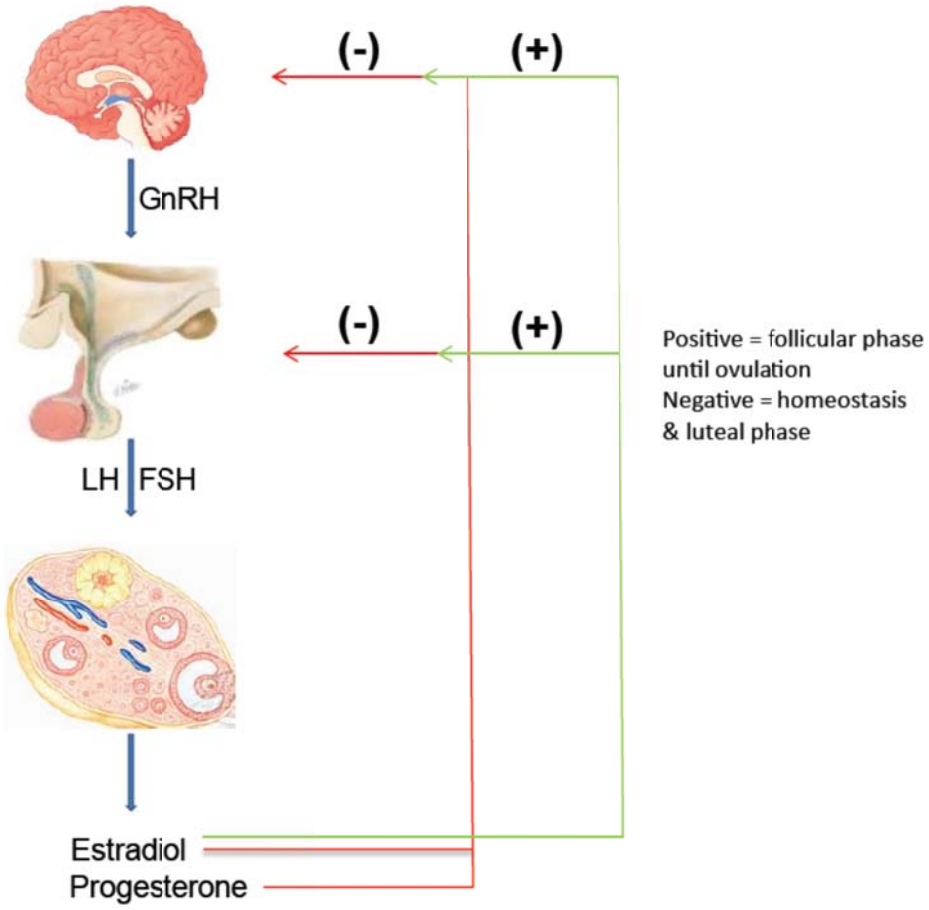


Figure 3. The Hypothalamic-Pituitary-Ovarian Axis. A schematic of this hormone axis shows both positive- and negative-feedback loops.

E_2 acts directly on both the hypothalamus and the anterior pituitary to inhibit hormone secretion. E_2 secreted from the ovaries inhibits secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus by binding to ERβ, thus suppressing GnRH neurons in the arcuate nucleus. E_2 also acts on the anterior pituitary by suppressing gene expression of the LH β-subunit, which inhibits the release of LH. Inhibition of hormone synthesis by the hypothalamus and pituitary decreases circulating concentrations of GnRH and LH, resulting in decreased production of E_2, which is typically derived from androstenedione by thecal cells of the ovary (Kovacs and Ojeda 2011). Inhibition of GnRH, LH and E_2 via this system completes the negative feedback loop, which can be carried out within minutes.

Positive feedback of the HPO axis is also controlled predominantly by ovarian E_2 but occurs only during the follicular development phase of the menstrual or estrous cycle. As follicles mature, they produce more E_2, which stimulates increased GnRH release from the hypothalamus and increased FSH and LH release from the anterior pituitary. Positive feedback continues throughout the follicular phase, building increasing concentrations of E_2, FSH and LH until an LH surge, which is responsible for ovulation, occurs. Promptly after ovulation, positive feedback ceases as E_2 secretion is inhibited by progesterone that is secreted by the corpus luteum. If pregnancy fails to occur, positive feedback of the HPO axis will resume when the luteal phase is completed. Contrary to the rapid response of the HPO axis' negative feedback system, a delay lasting several hours occurs before E_2 is able to induce the positive feedback system (Kovacs and Ojeda 2011).

1.4.5 Diseases of the ovary

A few ovarian diseases are responsible for the majority of ovarian dysfunction including polycystic ovary syndrome (PCOS), ovarian cancer, ovarian cysts and premature ovarian failure. Of these ovarian complications, PCOS is the most abundant endocrine related abnormality in women of reproductive age, affecting up to 18% of the female population (March et al. 2010). The etiology of PCOS is unknown, however those diagnosed with PCOS have increased prevalence of insulin resistance and obesity, which further increases risk of developing type II diabetes and cardiovascular disease (Shaw et al. 2008). Due to a multitude of phenotypes associated with PCOS including oligomenorrhoea/amenorrhoea, clinical or biochemical hyperandrogenism and polycystic ovaries detected by ultrasound, debate surrounds PCOS diagnostic criteria (Azziz et al. 2009). In addition to the increased risk of developing chronic diseases, PCOS is also responsible for reproductive dysfunction. The majority of women with PCOS (70-80%) experience oligomenorrhoea, infrequent menstruation; or amenorrhoea, absence of menstruation (Brassard et al. 2008). PCOS is the leading cause of annovulatory infertility striking up to 95% of women with annovulation (Teede et al. 2010). PCOS disrupts the HPO axis by increasing production and secretion of ovarian androgens, which impairs ovarian follicle development; excessive androgens induce other undesirable outcomes including excessive hair growth (Teede et al. 2010). Ovarian cysts can occur either as a result or independently from PCOS diagnosis. Ovarian cysts are fluid filled sacs found either in or on the surface of the ovary that can cause abdominal pain but often times are not related to any symptoms or negative health effects (Conway et al. 2014). Increased research and understanding of PCOS is needed to improve diagnostic and treatment implications. For example, because global increases in obesity rates are tightly related to

increasing PCOS rates, PCOS is a complicated public health issue resulting in an abundance of adverse health and reproductive outcomes (Shaw et al. 2008).

According to the American Cancer Society, ovarian cancer accounts for the most number of deaths out of all cancers of the female reproductive system. It is estimated that in 2015, more than 20,000 women will be diagnosed with ovarian cancer (American Cancer Society 2014). Like PCOS, the etiology of ovarian cancer is unknown; very few cases can be linked to genetic heredity leaving the majority of ovarian cancer cases sporadic (Doufekas and Olaitan 2014). Although epidemiological studies have revealed risk factors such as: age, genetic mutations in the BRCA1 and BRCA2 genes, previous endometriosis or breast cancer diagnosis, lack of parity, and others, these factors cannot fully explain the incidence of ovarian cancer observed today (Doufekas and Olaitan 2014). It is known that certain DNA or gene mutations can induce the growth of cancerous cells. The majority of the genetic mutations associated with ovarian cancer are not inherited but rather acquired throughout a woman's life. Genetic mutations can result from environmental exposures to radiation or carcinogenic chemicals; importantly, specific chemicals present in the environment have not yet been linked to mutations that result in ovarian cancer (American Cancer Society 2014).

Premature ovarian failure (POF) is defined as premature amenorrhea with elevated gonadotrophin levels observed under the age of 40; POF is commonly referred to as 'premature menopause' (Haller-Kikkatalo et al. 2015). Like PCOS, the cause of POF is unknown and many factors contribute to the diagnosis of this disease. Yet, diagnostic criteria vary and can involve assessments using ultrasound, bone mineral density, and serum E_2 and FSH levels (Conway et al. 1996). Given the heterogeneity of

POF diagnostic methods, it is extremely difficult to study its prevalence; therefore estimates of POF prevalence vary greatly and are thought to be inaccurate (Haller-Kikkatalo et al. 2015). POF contributes to female infertility, decreased circulating ovarian hormones, and increased risk of developing osteoporosis and heart disease (Lokkegaard et al. 2006).

1.4.6 Evidence of ovarian disruption linked to EDCs

Although limited, controlled animal and epidemiological data are available that suggest adverse female reproductive effects as a result of EDC exposure. One study found that female rats exposed to three different doses of BPA (0.25-0.62mg/kg, 2.5-6.2 mg/kg or 25.0-62.5 mg/kg) subcutaneously from postnatal day 1 to postnatal day 10 experienced hormonal and reproductive changes that manifested at 4-5 months of age. Rats exposed to the two highest doses had significantly increased testosterone and E_2 serum concentrations, which altered GnRH secretion in the hypothalamus; furthermore, all treatment groups had significantly decreased progesterone serum concentrations (Fernández et al. 2010). Animals treated with 25.0-62.5 mg/kg BPA had large numbers of ovarian cysts, cystic follicles and decreased numbers of total ovarian structures. These animals were also unable to ovulate or produce pups; animals treated with 2.5-6.2 mg/kg had significantly smaller litters when mated, consistent with decreased ovulatory responses at this dose as well. Collectively, these results suggest that administration of BPA at relatively high doses during neonatal development can result in symptoms consistent with PCOS and cause disruption of the HPO axis.

Another study observed disruption in follicular formation in rhesus monkeys exposed orally to 400 μg BPA/kg body weight/day late in the third trimester of fetal development when follicle formation occurs. This dose was demonstrated to produce peak serum levels of 2–5 ng/mL BPA, which is in the range of what has been reported in humans (Hunt et al. 2012). Animals treated daily with BPA had significantly increased numbers of multioocyte follicles (MOFs) and disrupted synaptic recombination between homologous chromosomes during meiosis. A large range of estrogenic exposures can induce MOFs (reviewed by (Pepling 2012)), and similar results showing MOFs induced by EDC exposures have been reported in mouse studies as well (Suzuki et al. 2002; Zhang et al. 2012). It is unclear how MOFs occur, however it is hypothesized that a delay in meiotic progression results in follicles that form before they undergo meiotic arrest and therefore are not ready for enclosure, allowing for the inclusion of multiple immature oocytes into one follicle. Reproductive outcomes as a result of MOFs are also unknown; follicular growth failure is suspected (Hunt et al. 2012).

Several epidemiological studies have associated EDC exposures with increased incidence of ovarian diseases. One study found that women diagnosed with PCOS had significantly higher serum BPA concentrations than similar women without PCOS (cases and controls, respectively) (Takeuchi et al. 2004). It was also observed that increasing serum androgen levels correlated with increasing serum BPA levels, suggesting that BPA concentration in blood is associated with a hyperandrogenic environment. Although the relationship between androgen and BPA concentrations in the blood is unclear, it is hypothesized that BPA is able to displace endogenous sex hormones from sex hormone binding globulin (SHBG) sites since it is a known SHBG ligand; this would lead to a

disruption in the androgen-to-estrogen ratio (Déchaud et al. 1999; Takeuchi et al. 2004). Similar results showing associations between increased BPA and androgen serum concentrations were found in another population of women diagnosed with PCOS (Kandaraki et al. 2010). It has been reported that BPA metabolism and excretion is down regulated by androgens through uridine diphosphate-glucuronosyl transferase activity in the liver (Takeuchi et al. 2006), providing further evidence for a complicated relationship between BPA and androgens.

Multiple human studies have observed adverse female reproductive health effects associated with exposure to other EDCs including PCBs, phthalates, and hormonally active pesticides (reviewed by (Mendola et al. 2008)). In short, these studies have found associations between EDC exposures and decreased age of menarche and puberty in girls, irregular menstrual cycles, irregular concentrations of circulating sex steroid hormones and adverse pregnancy outcomes (Mendola et al. 2008).

In addition to the cases discussed above, Peretz and colleagues reviewed all studies published between 2007-2013 that examined the impact of BPA on the male and female reproductive systems (Peretz et al. 2014). In summary, studies using various animal and cellular models as well as human data observed disturbances of the ovary in three categories: 1) oogenesis and ovarian follicle formation; 2) steroidogenesis in females; and 3) the quantity, quality and fertilization capacity of oocytes. Major findings from these three categories include: 1) Low dose BPA exposure decreased the number of primordial follicles in a dose-dependent manner in mice, induced changes in gene expression in germ cells in mice and disrupted meiosis in mice and macaques (Hunt et al. 2012; Lawson et al. 2011; Zhang et al. 2012). 2) Similar to the findings of Fernandez and

colleges, postnatal BPA exposure increased E_2, testosterone and progesterone levels in mouse serum (Fernández et al. 2010; Tan et al. 2013). 3) Human studies reveal correlations between serum BPA concentrations and oocyte maturation as well as decreased developmental potential of oocytes (Fujimoto et al. 2011). Increasing urinary BPA concentrations are also correlated with decreased number of mature oocytes and fertilized oocytes (Mok-Lin et al. 2010). Collectively these studies provide significant evidence that BPA exposure disrupts reproductive fitness of the ovary in laboratory animals, and similar relationships may be present in exposed humans.

1.5 Developmental Origins of Health and Disease (DOHaD)

The "developmental origins of health and disease" (DOHaD) hypothesis, also known as the Barker hypothesis, recognizes that exposure to environmental stressors during specific windows of development can result in permanent adverse health outcomes, often in the form of non-communicable diseases, later in life (Barker 1995; Barouki et al. 2012). The association between inadequacies during fetal life and later onset of disease was proposed by David Barker, who in the 1980s discovered increased risk of cardiovascular and metabolic diseases due to insufficient nutrition during development as a result of the Dutch famine (Barker 2007). Developmental plasticity allows organisms to adapt and respond to their surrounding environment in order to improve survival and reproductive capability (Barouki et al. 2012). Differentiation and development of cells and tissues occurs during time frames of development that are specific to the particular organ or tissue that is being formed. Exposure to a variety of environmental factors including drugs, inadequate nutrition, stress, environmental

chemicals and other environmental factors during "critical windows of development" can alter cellular and epigenetic programming of specific tissues and organs including the ovary (Heijmans et al. 2009; Heindel and Vandenberg 2015).

Many chemicals are able to cross the placental barrier and enter the fetal circulation and/or the surrounding fetal environment including the amniotic fluid; therefore the developing organism can experience exposure to these compounds *in utero* (Arbuckle 2010; Woodruff et al. 2011). Furthermore, as mentioned previously, many environmental chemicals are highly lipophilic molecules that can bioaccumulate within adipose tissue and can be transferred to developing offspring through breast milk, resulting in exposure during critical postnatal periods of development. Persistent environmental chemicals found in breast milk globally have been reviewed in depth by several reports (Fürst 2006; Solomon and Weiss 2002). A recent study revealed that at least 100 chemicals are present in the cord blood of infants, which suggests that fetal exposure to an array of environmental chemicals including EDCs occurs during fetal development (Unuvar and Buyukgebiz 2012). Many studies have been performed which link developmental exposure to various EDCs to adverse female reproductive health outcomes (reviewed by (Crain et al. 2008)). The DOHaD hypothesis has been applied to explain the associations between developmental exposures to EDCs and increasing rates of reproductive diseases and dysfunctions including subfecundity, PCOS, uterine fibromyoma and some types of reproductive cancers (Barouki et al. 2012).

The DOHaD concept has become a well-recognized framework for modeling how environmental chemicals and other environmental factors could contribute to the increasing rates of chronic disease that the world's human populations are experiencing

including type II diabetes, obesity, cardiovascular disease and various types of cancer (Barouki et al. 2012). Currently, non-communicable diseases are the most prominent causes of death in industrialized countries, making the study of environmental chemicals an important public health issue. Importantly, the DOHaD hypothesis provides new strategies and implications to improve research and disease prevention measures by creating a focus on the timing of disease (Barouki et al. 2012; Heindel and Vandenberg 2015); if DOHaD is sufficient to explain the associations between environmental chemicals and later life diseases, limiting exposures to environmental toxins during pregnancy and childhood could prevent chronic diseases in adulthood thus, decreasing the global burden of non-communicable diseases.

CHAPTER 2

PROJECT RATIONALE

Throughout the past several decades an overwhelming amount of scientific data has been published regarding EDC exposures and related negative health outcomes. EDCs differ greatly from other classes of environmental chemicals, posing complex questions regarding the understanding of biological and physiological actions and effects of EDCs, and creating debate in the scientific community. Current risk assessment methods assess chemicals as if they follow a classic linear dose-response model, assuming that low doses are less harmful than higher doses. At least some of the time, EDCs defy this expectation; diverse adverse health effects are often induced at low doses.

BPS and TBBPA are ubiquitous in the environment and are structurally similar to the EDC BPA, which has an array of known negative health outcomes associated with its exposure. The use of these chemicals is largely unregulated and human populations globally are exposed on a daily basis. The public health and environmental concern at hand is the potential of negative health effects to arise due to BPS and TBBPA exposure. When looking at this issue from a DOHaD lens, this issue is especially concerning to pregnant women and children who face exposure during critical developmental windows.

In this report, I analyze the results observed from exposing mice perinatally to BPS and TBBPA by looking at developmental endpoints relevant to ovarian development and a small number of additional estrogen-sensitive endpoints. The chemicals BPS and TBBPA are hypothesized to act as EDCs based on previous exposure reports and their similarity in structure to BPA, a well-studied EDC; this is the major rationale for the selection of BPS and TBBPA for this study.

The ovary was chosen as the organ of study for two main reasons: 1) The ovary is an organ that is highly sensitive to hormones and EDCs therefore, if hormonal disruption is taking place, we would expect to see changes within the ovary. 2) The ovary is involved in the complex HPO axis, which provides strict control of multiple hormones via positive and negative feedback systems. If hormone disruption occurs at any of the three organs involved in the HPO axis (hypothalamus, pituitary, ovaries), we would expect to see changes in the ovary. Observed endpoints were carefully selected in order to observe potential changes in the ovary at a gross, cellular and molecular level. Gross alterations were assessed during necropsy by looking for abnormalities such as ovarian cysts and measuring uterine weight. Changes at the cellular level were assessed through histological analysis of ovarian sections. Finally, immunohistochemistry and qPCR provided data regarding changes in the ovary at a molecular level via protein and gene expression analysis.

2.1 Hypothesis Statement

Based on previous studies that link developmental exposure to similarly structured environmental EDCs to adverse female reproductive outcomes, I hypothesize that developmental exposure to BPS and TBBPA will induce changes in estrogen dependent endpoints such as ovarian development, with effects observed on a cellular and molecular level. This supports an additional hypothesis that BPS and TBBPA are capable of disturbing ovarian hormone functions via estrogen dependent pathways and should be considered EDCs.

CHAPER 3

METHODS AND MATERIALS

3.1 Animals

Adult outbred CD-1 mice of both sexes were obtained from Charles River Breeding Laboratories (Raleigh, NC). The CD-1 strain has previously been shown to be a sensitive model for the use of reproductive endocrine disruption studies (Peretz et al. 2013). All procedures with mice were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Massachusetts, Amherst. Two females and one male were housed together in a ventilated cage until pregnancy was confirmed via the presence of a vaginal plug. Pregnant females were housed with up to two other females of the same dosing group until pregnancy day 17. On pregnancy day 18, each pregnant female was separated into her own cage in preparation for delivery. A 0600–1800 hours light cycle and a controlled temperature of 25–27 °C was followed for the entirety of the experiment. Standard rodent chow and water were provided for ad libitum consumption.

3.2 Chemical administration

Females were randomly assigned to exposure groups using statistical methods to ensure similar body weights across treatment groups. Females were ear-tagged upon arrival to allow for their identification; identification tags were coded so that experimenters were blind to treatment throughout the experiment.

The day that a vaginal plug was observed was considered embryonic day 1 (E1). Pregnant females were orally dosed with 200μg/kg of body weight/day of BPS in corn oil

(n=5), or 200µg/kg/day of TBBPA in corn oil (n=5), or with tocopherol-stripped corn oil alone (vehicle control, n=5). Mice were trained to drink these substances (1 µl per gram body weight) from the end of a pipette. Pregnant and lactating mice were treated from pregnancy day 8 until lactational day 20 (Figure 4).

Additionally, two female offspring (F1 generation) were selected from each litter for additional treatments. One offspring was dosed orally by pipette with 1µg/kg/day of ethinyl estradiol in tocopherol-stripped corn oil from postnatal day (PND) 19 to PND21. The other offspring was treated with tocopherol-stripped corn oil alone. These two female siblings were killed on PND22 (Figure 4). All other pups were weaned at PND 21 and separated by sex into cages with others of the same treatment group.

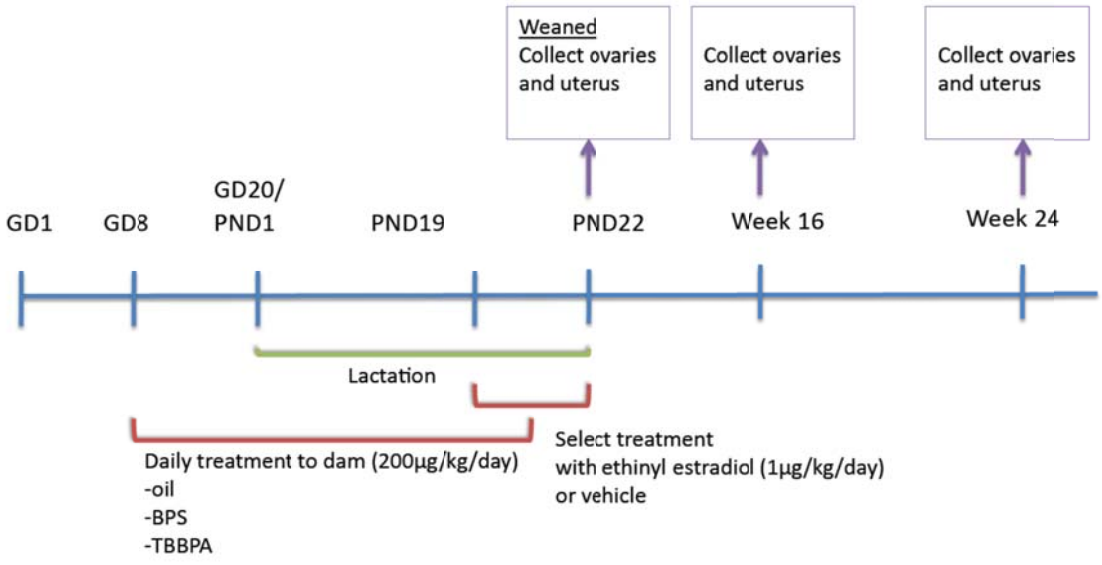


Figure 4. Experimental schematic. The timing of developmental exposures to TBBPA and BPS are indicated, as well as the timing of the pre-pubertal EE_2 challenge.

3.3 Tissue and body weight collection

Only female pups were used in this experiment. As mentioned above, two female pups from each litter were sacrificed on PND22 (one treated with ethinyl estradiol, and one treated with corn oil, from PND19-PND21). From these females, ovarian and uterine tissues were collected; body weight was also measured at time of sacrifice. At 16 weeks of age, 1-2 female pups from each litter were sacrificed, ovarian and uterine tissues were observed for gross abnormalities, then collected as described below; uterine weight and body weight were also measured. The remaining female pups were sacrificed at 24 weeks of age, and ovarian and uterine tissues were collected and uterine weight was measured. Individual body weight was again recorded on the day of euthanasia. At all ages, one ovary and one horn of the uterus from each animal were frozen at -80°C for qPCR analysis. The second ovary and the remainder of the uterus were fixed in neutral buffered formalin for histological analysis.

3.4 Behavioral assessments

Females were assessed in an Open Field test at three different ages: PND22-24 (pre-puberty), 15 weeks, and 23 weeks of age. Briefly, the Open Field test involves the placement of females in a clear plexiglass cage sized 40 cm x 40 cm x 40 cm. Females were observed for 5 minute periods without experimenter intervention. At all three ages, the Noldus Ethovision software was used to record basic parameters including: distance traveled, average speed, and cumulative duration in the center of the apparatus. During the 15 week and 23 week experiments, additional data was collected by hand including

number of rears on walls, number of rears in the center of the open field, number of crosses through the center of the apparatus, and time spent grooming.

3.5 Analysis of follicle formation

To characterize follicle formation, fixed ovaries from PND22 and week 16 females were processed through a series of alcohols and embedded in paraffin. Paraffin blocks were sectioned at 5μm on a Fisher rotary microtome and mounted on positively-charged glass slides. Sections were processed through a series of xylene and ethanol, stained with hematoxylin and eosin, dehydrated, and coverslipped with permanent mounting medium. Images were collected on a Zeiss AxioImager Inverted Microscope with ZEN imaging software and a Zeiss high resolution color camera with a 20x EpiPlan Objective.

To score follicles, every tenth section throughout the entire ovary was analyzed, corresponding to one section per 50μm of ovary. In addition to the analysis of sections from the entire ovary, four consecutive sections from the medullary region were analyzed separately to minimize statistical error due to variation between ovarian sizes. Sections were coded, allowing for scoring by a blinded observer. Follicles were scored using methods modified from those described previously (Hunt et al., 2012) and categorized into five categories depending on the stage of follicle development: primordial, primary 1, primary 2, secondary and antral (Figure 2). Follicles were scored as: “primordial” if the nuclei were along the cortex of the ovary and had no granulosa cell formation; “primary 1” if they had one layer of granulosa cells around the nucleus; “primary 2” if they had two layers of granulosa cells but no theca cells around the nucleus; “secondary”

if they had more than two layers of granulosa cells and at least one layer of theca cells around the follicle; and "antral" if they had the same criteria of a secondary follicle with the addition of a follicular fluid-filled antrum. Follicles of these various stages are shown in Figure 2**.** The total number of oocytes, total number of follicles, and total number of corpus lutea (when appropriate) were also counted for each section of ovary analyzed.

3.6 Immunohistochemistry

3.6.1 Technical methods

For each primary antibody of interest (Ki67, ERα), one 5μm ovarian tissue section per female was analyzed. In total, sections were analyzed from one female per litter from each of the following groups: 1) at PND22 after three days' treatment with oil; 2) at PND22 after three days' treatment with ethinyl estradiol; and 3) at 16 weeks of age. Ki67 antibodies were obtained from Vector Labs (rabbit polyclonal, Cat# VP-RM04) and ERα antibodies were obtained from Millipore (rabbit polyclonal, Cat# 06-935). Antibodies were first optimized for ovarian tissue and the optimum concentration found for Ki67 and ERα was determined to be 1:1000.

For immunohistochemical analysis, a protocol developed previously was modified. Briefly, a series of xylene and ethanol washes was used to remove the paraffin from the tissue. For antigen retrieval, microwave treatment paired with citric acid buffer was used followed by a step for quenching endogenous peroxidase activity. Non-specific antibody sites were blocked using normal goat serum 1:20 in 1.5% milk. After overnight incubation with the primary antibody at 4°C, the tissue was incubated with the biotin labeled anti-rabbit secondary antibody (Abcam Cat# ab64256) for one hour at room

temperature, and the reaction was developed with streptavidin peroxidase complex (Abcam Cat# ab64269). Finally, a color-change reaction was obtained using DAB (3,3-diaminobenzidine, Abcam Cat# ab64238) and tissue was counterstained with hemotoxylin before being cover-slipped with permanent mounting media.

3.6.2 Analysis

Each sample was imaged with a Zeiss AxioImager Inverted Microscope with a 20x EpiPlan Objective with ZEN imaging software and a Zeiss high-resolution color camera. All analyses were conducted by an observer blind to treatment. When possible, two of each follicle type with granulosa cells discussed above in section 3.5 (primary 1, primary 2, secondary, antral) were analyzed. Follicles were analyzed by counting the number positive/brown cells (both granulosa and theca cells were counted) and by counting the number of negative/blue cells (again, both granulosa and theca cells were counted). The total percentage of granulosa and theca cells that were positive for the specific antibody being tested was then calculated (# positive / total number counted x 100). Corpus luteal cells were also analyzed (when appropriate) by counting the number of positive/brown and negative/blue luteal cells. Total percent positive luteal cells were calculated.

3.7 qPCR

3.7.1 RNA isolation from tissue

Ovaries collected from female mice described in Section 3.3 were homogenized individually in Trizol to lyse cells and release RNA. Chloroform was added to each sample and samples were then spun in a microcentrifuge at 4^{o} for 15 minutes to separate

the RNA from Trizol and RNA was precipitated with isopropanol, washed twice with 75% ethanol, and suspended in 50µl of RNase-free water. The RNA concentration and purity (260/280) was determined by Nano-drop. Samples were stored at -80^0.

3.7.2 cDNA synthesis

Reactions were performed in PCR tubes on ice using the iScript Reverse Transcription Supermix for RT-qPCR kit (Cat 170-884, BioRad, Hercules, CA). Three categories of reactions were carried out. The first category contained RNA samples that underwent reverse transcription to produce cDNA. This reaction utilized 1µg of RNA from step 3.7.1 and 4µg of 5x iScript RT supermix. The second category was a no reverse transcriptase (NRT) control, to control for genomic DNA that may have contaminated the sample. The NRT control contained 1µg of RNA from step 3.7.1 and 4µg of 5x iScript RT supermix no RT control. A NRT control was made for each sample of RNA. The third category was a no template (NT) control to control for the possible self-amplification of primers. The NT control contained 1µg of reverse transcriptase. Only two random NT controls were made since they were reused as controls. All samples were run on a BioRad thermal cycler using standard temperature and time protocols: 25^o for 5 minutes, 42^o for 30 minutes, 85^o for 5 minutes, 4^o for ∞. Samples were stored at -80^o.

3.7.3 Primer design

The UCSC genome browser (genome.ucsc.edu) for mouse (*Mus musculus*) was used to select nucleotide sequences of two sequential exons. The forward primer was designed to sit on a selected exon of the gene of interest and the reverse primer to sit on the very next exon downstream of the first selected exon. Primers were developed using the Primer3Plus database (http://www.bioinformatics.nl/cgi-

bin/primer3plus/primer3plus.cgi/). The exon sequences recorded from the USCS genome browers were used to generate forward and reverse primers. After primers were generated in Primer3Plus, primer sequences were put into the UCSC BLAT search genome function to verify that the primer sequences were 100% specific and that they lie on the correct chromosome. Primer sequences are listed in Table 1 and are shown from 5' to 3'.

3.7.4 qPCR

All reactions were assembled in eppendorf tubes on ice. For each sample of RNA, a RT and NRT control sample were analyzed. For each qPCR run, one NT control sample was analyzed. First, yeast tRNA was prepared by diluting 50ug/ml stock solution to a 1:200 dilution with dH_20. Yeast tRNA was used to prevent cDNA from sticking to the sides of the eppendorf tube. Next, cDNA samples from 3.7.2 were diluted 1:10 into yeast tRNA made in the previous step. The supermix solution was then made by combining 10µl iTaq Universal SYBR Green Supermix (Cat 172-5124, BioRad) and 3µl of dH_20 per sample. The supermix solution was then evenly distributed into each of the tubes containing cDNA and yeast tRNA, 15µl of this solution was distributed into new eppendorf tubes (enough tubes for each of the primers being analyzed.) The PCR reaction mix contained 5µl of the desired primer solution from 3.7.3 (containing both forward and reverse primers) and 15µl of the supermix solution, resulting in 20µl of total solution for each sample. Samples were briefly vortexed and spun in a centrifuge to condense all liquid to the bottom of the tube. In a 384 well PCR plate, 5µl of the PCR reaction mix was pipetted into three consecutive wells and the plate was sealed with adhesive film. PCR reactions were carried out with a BioRad CFX384 real time cycler using standard temperature and time protocols.

Table 1. Primer sequences used for qPCR analysis

Gene	Forward primer sequence	Reverse primer sequence
Androgen receptor/AR	AAG ACC TGC CTG ATC TGT GG	TCG TTT CTG CTG GCA CAT AG
Epidermal growth factor receptor/EGFR	TCT TCA AGG ATG TGA AGT GTG	TGT ACG CTT TCG AAC AAT GT
Estrogen Receptorα/Esr1	TGC AAT GAC TAT GCC TCT GG	CTC CGG TTC TTG TCA ATG GT
Estrogen Receptorβ/Esr2	ACT GCC AAT CAT CGC TTC TC	AGT AAC AGG GCT GGC ACA AC
Insulin like Growth Factor 1/IGF1	GGA CCA GAG ACC CTT TGC GGG G	GGC TGC TTT TGT AGG CTT CAG TGG
Progesterone Receptor/PR	CCA GCA TGT CGT CTG AGA AA	ACC ACA TCA GGC TCA ATG CT

3.7.5 Analysis

cDNA amplification data was analyzed using Bio-Rad CFX manager software. Samples were assured for quality of target cDNA amplification with the use of the melting curve tool and amplification/Cq values. For each sample, the mean Cq value was calculated for the three PCR wells that contained the sample. The mean sample Cq value/ level of gene expression was then normalized to mean Cq value/expression level of the reference gene (B2M) from the same sample, resulting in a relative quantification value (see, formula 1). Relative quantification values were then statistically assessed based on animal treatment group.

Formula 1: Relative quantification value for the normalization of target gene expression

$$\text{Relative quantification} = 2^{-\Delta Cq}$$

3.8 Statistical analysis

All statistical analysis was carried out with the use of *SPSS* statistical software version 22. Effect of chemical treatment was assessed for mean uterine weights, histological analysis of the ovary, immunohistochemical analyses in the ovary, and ovarian gene expression with the use of an ANOVA test followed by a Bonferroni Post Hoc test. Comparisons between oil vehicle and ethinyl estradiol treated females at PND22 were conducted with independent samples T-tests. Graphs were made in excel, and all graphs show mean ± SEM. Results were considered significant at $p<0.05$. Statistical trends were identified as $p>0.05$ but <0.10.

CHAPTER 4

RESULTS

4.1 Body and uterine weight

Body weight is a standard measure of animal health. In order to measure any significant decrease in animal health potentially induced by chemical treatment, body weight was measured before sacrifice at PND 22, 16 weeks and 24 weeks of age. No notable changes in animal body weights were observed for either of the chemical treatment groups in comparison to the control group or between chemical treatment groups at any of the three measured ages (data not shown).

Wet (unblotted) uterine weight is a standard measure of estrogencity. To measure estrogenic effects induced by chemical exposure, wet uterine weight was recorded at 16 and 24 weeks of age. Although we did not measure uterine weight at PND22, other measures of estrogenicity (i.e. height of the uterine epithelium, expression of lactoferrin, etc.) are being conducted in the Vandenberg laboratory by other researchers; the data from these analyses are forthcoming. At both 16 and 24 weeks of age, no significant changes in uterine weight were observed between the control and chemical treatment groups. However, an ANOVA with Bonferroni post hoc test revealed a significant difference between the BPS and TBBPA treated groups at 16 weeks of age (Figure 5A), with mean uterine weights of BPS treated animals (0.169 g) that were significantly heavier than the mean uterine weight of TBBPA treated animals (0.119g) ($p=0.02$). No significant changes in uterine weight were observed at 24 weeks of age (Figure 5B).

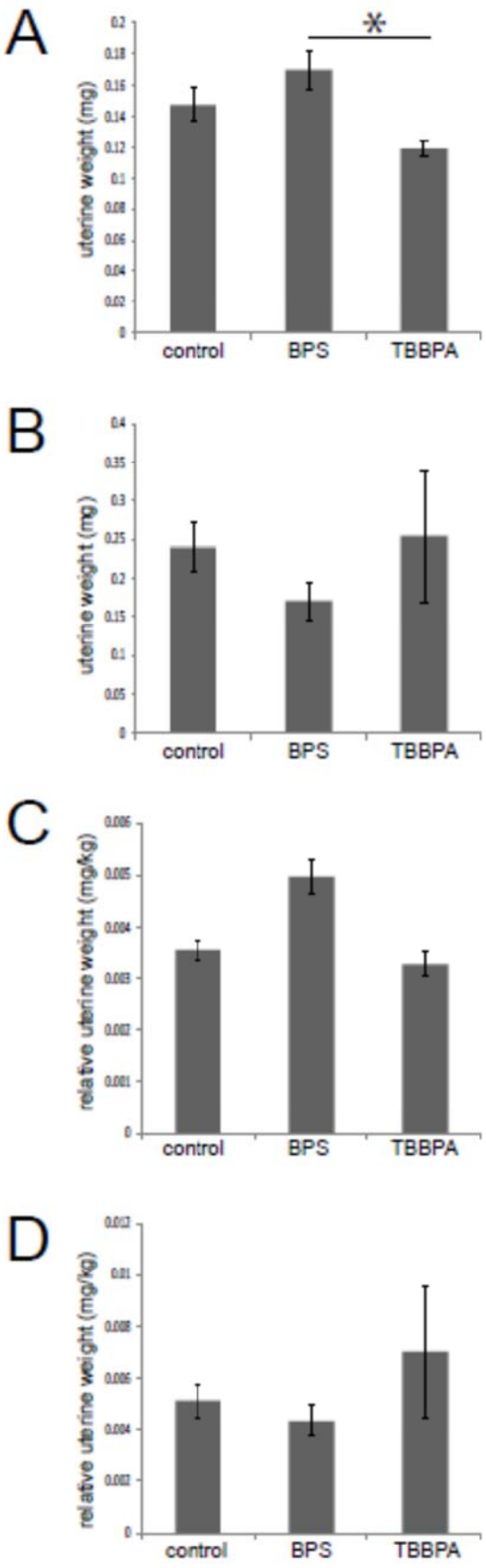


Figure 5. Effects of developmental BPS and TBBPA exposures on uterine weight and relative uterine weight in adulthood. A) Uterine weight at week 16. B) Uterine weight at week 24. C) Relative uterine weight at week 16. D) Relative uterine weight at week 24. *p<0.05, Bonferroni posthoc test.

Since body weight was variable between individual animals, uterine weight: body weight ratio was calculated. No significant differences were observed between treatment groups for relative uterine weight at 16 or 24 weeks of age (Figure 5C,D).

4.2 Ovarian cysts

Ovarian cysts were determined based on gross morphology of the ovary during necropsy. At PND22, cysts were not observed in any females in any treatment group. However, by 16 weeks, cysts were observed in 3/8 females treated with BPS, an increase compared to the frequency of cysts in the vehicle-treated controls (0/5, see Table 2). At 24 weeks of age, cysts were observed in all treatment groups but were more common in the TBBPA and BPS-treated females compared to controls (Table 2).

Table 2. Frequency of ovarian cysts in females treated perinatally with test chemicals

	Controls	TBBPA	BPS
% with ovarian cysts at 16 weeks of age (n)	0% (5)	0% (6)	37.5% (8)
% with ovarian cysts at 24 weeks of age (n)	18.2% (11)	28.6% (7)	25% (8)

4.3 Open field behavior assessment

To assess potential changes in sexually dimorphic behaviors induced by chemical exposure, female offspring were assessed at 15 and 23 weeks of age for a variety of previously validated open field behaviors that provide information regarding sexual

dimorphic behavior. Animals were scored by hand based on the following criteria in the open field test: number of rears on walls, number of rears in the center of the open field, number of crosses through the center of the apparatus, and time spent grooming (described in 3.4). Additional criteria were scored by an automated system, Noldus software, at PND22, week 15, and week 23 and included the total distance covered (cm) by each mouse during the 5 minute trial, their mean velocity (cm/s), the percentage of time the animal was moving, and the percentage of time the animal spent in a 5cm by 5cm square in the center of the open field.

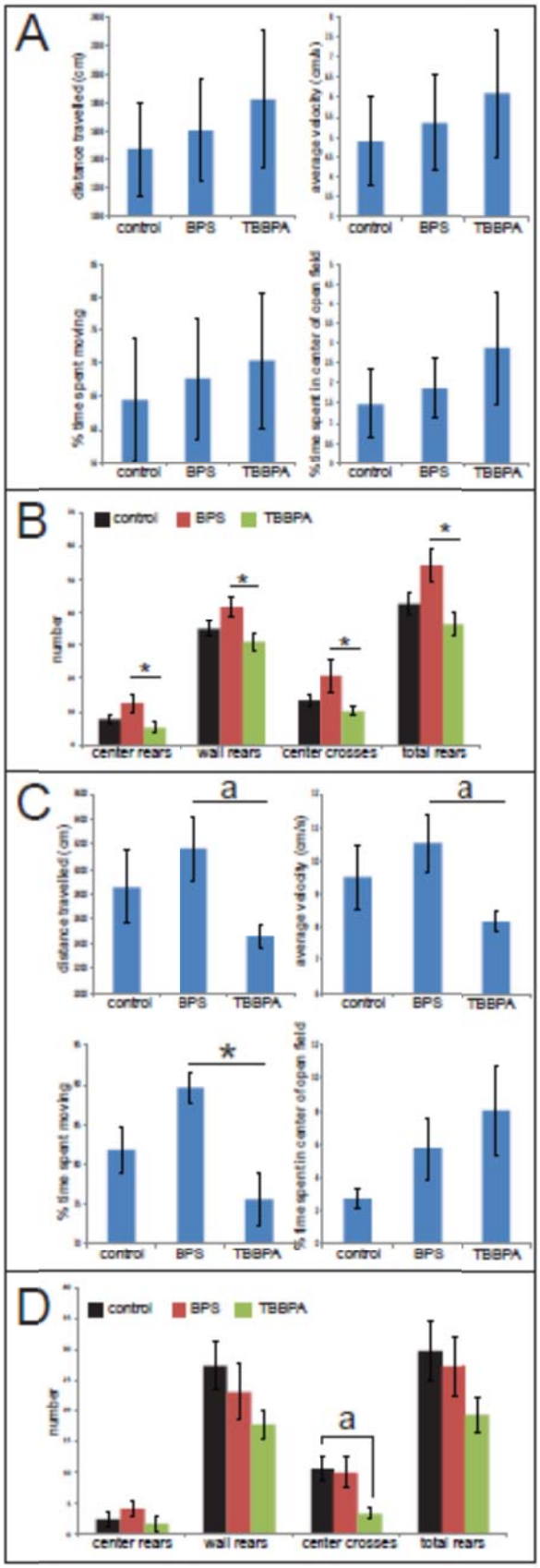
4.3.1 PND 22

The Noldus software was used to measure four aspects of behavior in the open field (Figure 6A). At this age, there were no significant differences between any treatment groups.

4.3.2 Week 15

Significant changes in behavior were observed in four categories: center rears ($p=0.032$), wall rears ($p=0.029$), center crosses ($p=0.041$) and total rears ($p=0.010$). For all four categories, BPS-treated animals had increased measures compared to controls and TBBPA had decreased measures compared to controls, although these differences were not statistically significant (Figure 6B). Yet, in all four categories the specific behavior was significantly increased in BPS treated animals compared to TBBPA treated animals.

Using the Noldus software, four quantitative measures were compared between the three treatment groups, and similar trends were observed for distance travelled, average velocity, and time spent moving: BPS treated females were typically more active than controls, and TBBPA-treated females were typically less active than controls (Figure



6C). Although significant differences were not observed for either chemically-treated group compared to controls, BPS and TBBPA were significantly different from each other.

Figure 6. Effects of developmental BPS and TBBPA exposures on behaviors in the open field. A) Behavioral parameters measured by the Noldus software at PND22-24. B) Parameters measured by hand in females at 15 weeks of age. C) Parameters measured by the Noldus software at 15 weeks of age. D) Parameters measured by hand at 23 weeks of age. In all panels, [a] indicates a p-value between 0.05 and 0.09, and * $p<0.05$, Bonferroni posthoc test.

4.3.3 Week 23

No significant changes in behavior for the open field test were observed at 23 weeks of age between any treatment groups. There was a trend for a decrease in the number of center crosses in the TBBPA-treated animals compared to control animals although this was not statistically significant (Bonferroni, control vs TBBPA, p=0.092, Figure 6D). The Noldus data has been collected but will be analyzed in the future.

4.4 Ovarian follicle formation

Based on the hypothesis that BPS and TBBPA are able to induce estrogenic effects and thus induce premature ovarian development, we compared morphology of ovarian follicles at PND 22 and 16 weeks of age from animals developmentally exposed to vehicle, BPS and TBBPA. The method for follicle scoring is discussed in depth in section 3.5.

4.4.1 PND 22

At PND 22, the follicle-score categories assessed included: primordial follicles, primary 1 follicles, primary 2 follicles, secondary follicles and antral follicles (described in detail in section 3.5). Follicles from every tenth section of the entire ovary as well as follicles from only four sections of the medullary region were scored separately to account for variability in ovarian size. Significant changes observed in follicle category numbers from the entire ovary are shown in Figure 7, and from four sections in Figure 8.

Results from the analysis of every tenth section of the ovary are described as follows: EE_2 induced a significant increase in the number of oocytes throughout the entire ovary in control (p=0.037) and TBBPA (p=0.001) treated animals (Figure 7A), and

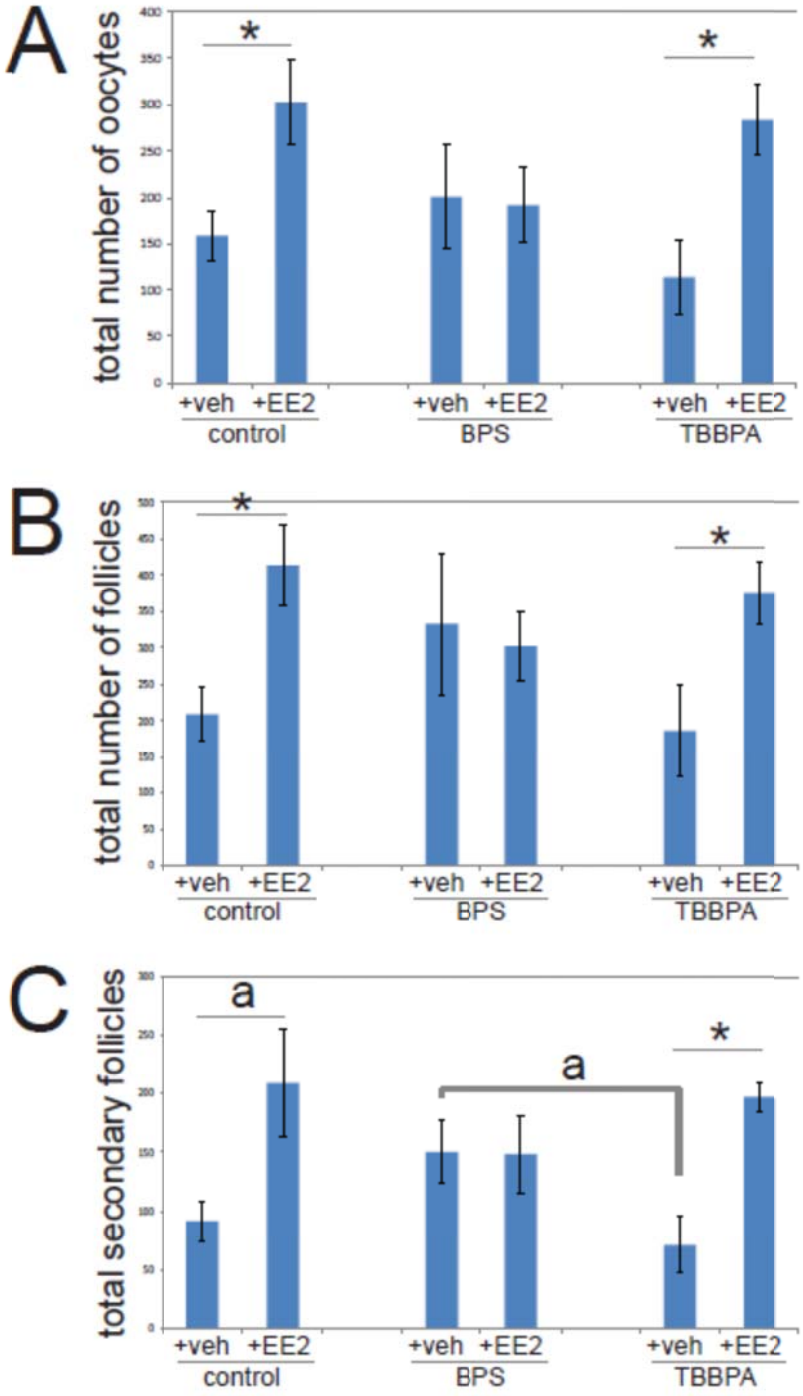


Figure 7. Ovarian follicular parameters measured in entire ovaries at PND22 with and without an EE_2 challenge. A) Total number of oocytes. B) Total number of follicles. C) Total number of secondary follicles. In all panels, [a] indicates a p-value between 0.05 and 0.09, and * $p<0.05$, Bonferroni posthoc test.

the number of follicles in control (p=0.024) and TBBPA (p=0.038) treated animals (Figure 7B). These effects induced by EE_2 were not observed in BPS treated animals. EE_2 also induced a significant increase in the number of total secondary follicles in TBBPA treated animals (p=0.001) and in control animals (p=0.053, Figure 7C). Again, EE_2 failed to increase the number of secondary follicles in BPS treated animals. An ANOVA revealed differences in the number of secondary follicles between control, BPS and TBBPA vehicle treated animals (p=0.082, Figure 7C). However, differences between specific treatment pairs were not identified. In summary, the EE_2 challenge increased the number of total oocytes, secondary follicles and total number of follicles throughout the entire ovary in control and TBBPA, but not BPS, treated animals.

Results from analysis of four consecutive sections from the medullary region of the ovary are described as follows: An increase in oocytes in four sections was observed for control animals (p=0.054) treated with EE_2 (Figure 8A). An ANOVA with Bonferroni post hoc analysis identified a significant difference between the number of oocytes in four sections between EE_2 challenged control and BPS treated animals (p=0.026, Figure 8A); the number of oocytes was significantly greater in the control group compared to BPS-treated females. Similar to the results above, EE_2 challenge also induced significant increases in total number of follicles in four sections of control (p=0.04) and TBBPA (p=0.013) treated animals (Figure 8B), and the number of secondary follicles in four sections of control (p=0.016) and TBBPA (p=0.01) treated animals (Figure 8C). An ANOVA revealed a trend in changes to the number of secondary follicles in four sections between the three vehicle treatment groups (p=0.061) and a difference in the number of total follicles in four sections between the three EE_2 treatment groups (p=0.092) however,

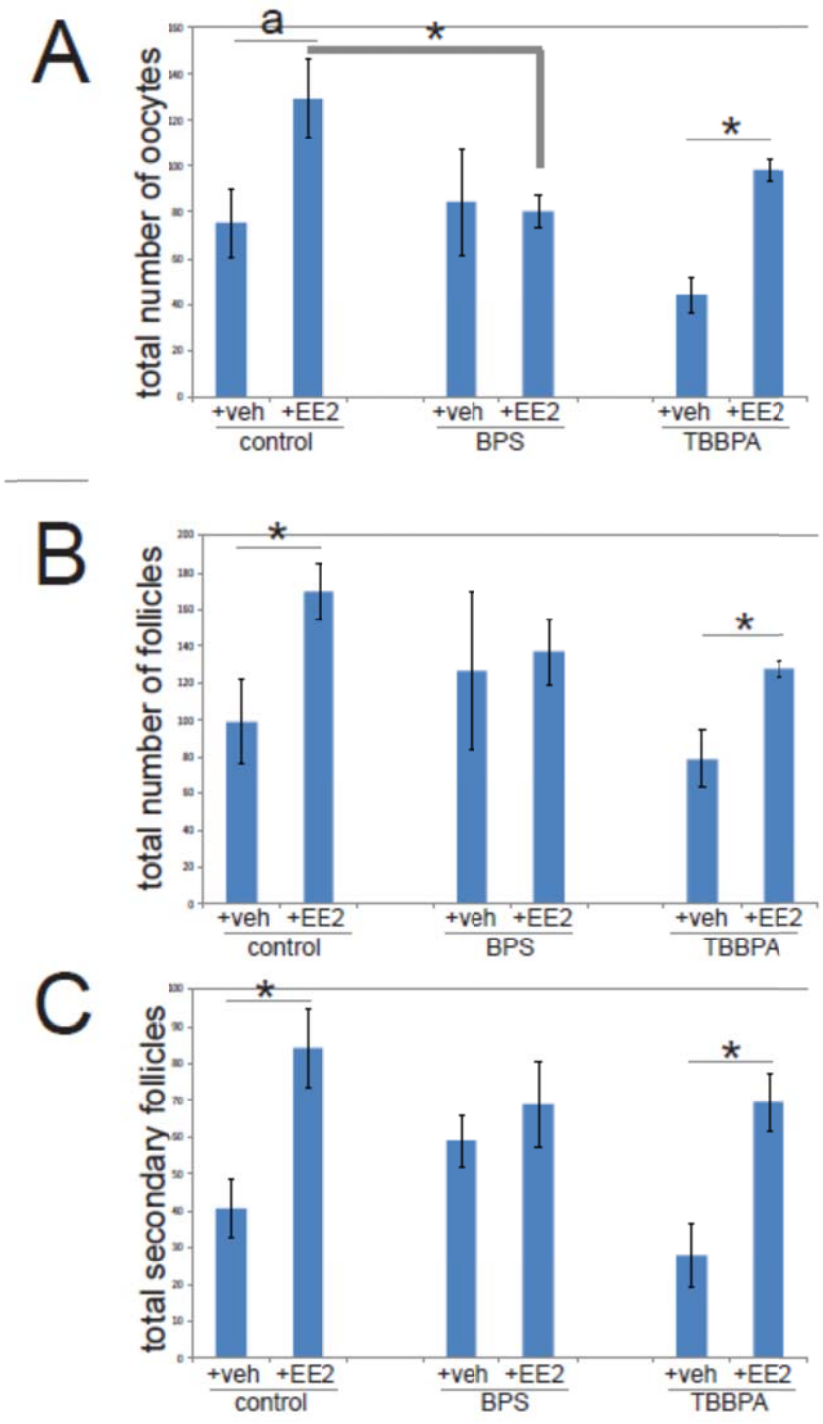


Figure 8. Ovarian follicular parameters measured in four medullary sections at PND22 with and without an EE_2 challenge. A) Total number of oocytes. B) Total number of follicles. C) Total number of secondary follicles. In all panels, [a] indicates a p-value between 0.05 and 0.09, and * $p<0.05$, Bonferroni posthoc test.

differences between specific treatment pairs could not be identified with a Bonferoni post hoc test. Additionally, a non-significant difference was identified in the number of primary 1 follicles in four sections of EE_2 challenged control and TBBPA treated animals ($p=0.053$, data not shown); the number of primary 1 follicles was decreased in the TBBPA treated group.

Finally, because prior studies with BPA reported an effect of this chemical on multi-oocyte follicles (MOFs), we counted follicles that contained two or more oocytes within the bounds of the surrounding granulosa cells (Figure 9). MOFs were not observed in control animals, with or without an EE_2 challenge. Further, only one animal exposed to BPS during the perinatal period had MOFs (Table 3). In contrast, four animals exposed to TBBPA during development had visible MOFs; three of these animals had received the EE_2 challenge (Table 3).

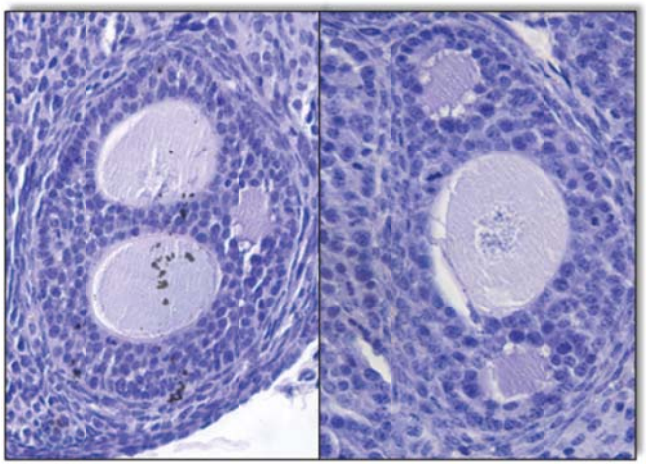


Figure 9. Examples of MOFs.

Table 3. Number of animals with MOFs visible in histological sections at PND22.

	Control	TBBPA	BPS
Postnatal vehicle	0/4	1/5	1/4
Postnatal EE_2	0/4	3/5	0/4

4.4.2 Week 16

At 16 weeks of age, follicle-score categories assessed included: primordial follicles, primary 1 follicles, primary 2 follicles, secondary follicles, antral follicles and corpus lutea (described in detail in section 2.5, see also Figure 2). Follicles from every tenth section of the entire ovary as well as follicles from only four sections of the medullary region were scored separately to account for variability in ovarian size. No significant or notable changes in follicle scores were observed in relation to treatment at 16 weeks of age for assessment of every tenth section or four sections of the ovary (data not shown).

Again, we examined the 16 week ovaries for the presence of MOFs. At this age, MOFs were observed in all treatment groups including the controls (Table 4).

Table 4. Number of animals with MOFs visible in histological sections at week 16.

Control	TBBPA	BPS
2/4	2/4	1/5

4.5 Immunohistochemical analysis of the ovary

Next, ovarian tissue was assessed for changes in expression of proteins crucial in estrogen function and cell proliferation (ERα and Ki67). Changes in expression of these proteins could potentially describe treatment related changes observed in follicle formation assessments. Ovarian tissue sections were analyzed for Ki67 and ERα expression levels based on follicle type at PND 22 and postnatal week 16 (described in 2.6, see Figure 10).

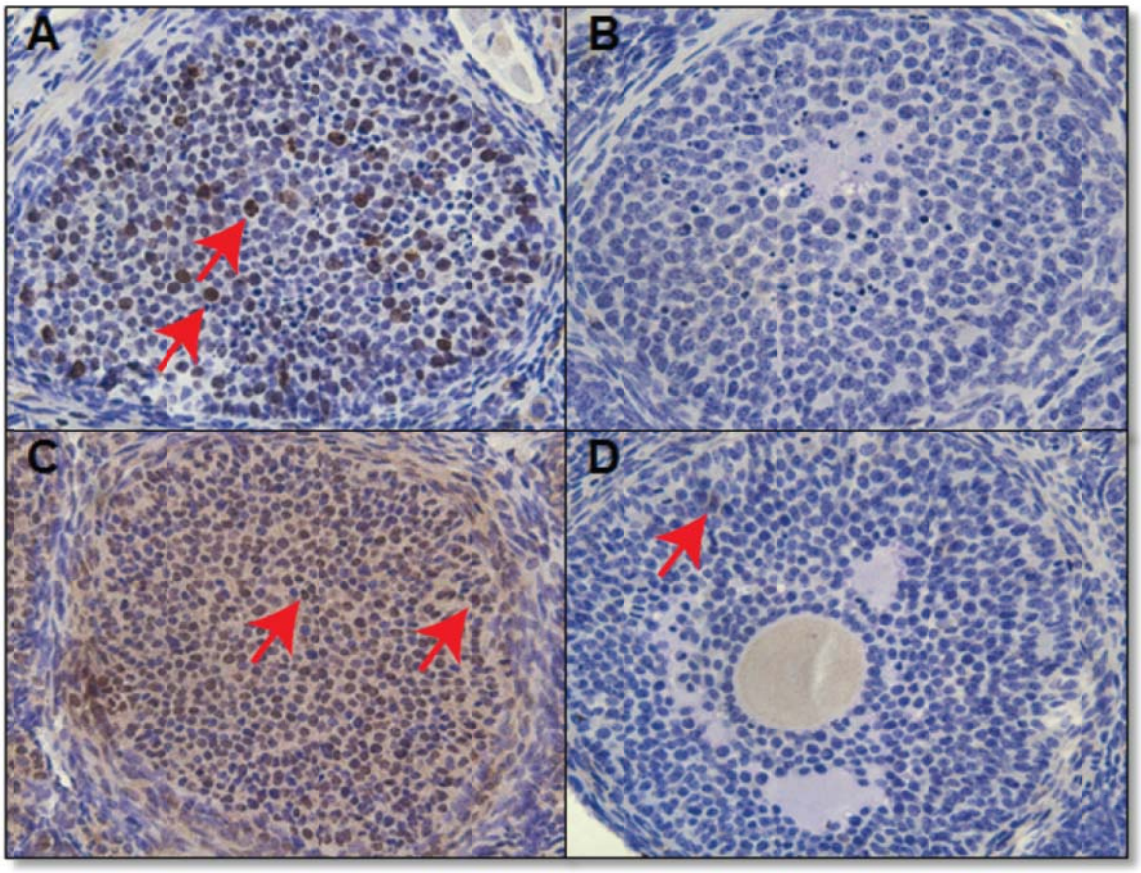


Figure 10. Immunohistochemical analyses of Ki67 and ERα expression. A-B) High and low expression of Ki67. C-D) High and low expression of ERα. In all sections, red arrows are indicating positive cells.

4.5.1 PND 22

EE_2 induced a significant increase in Ki67 expression in primary 2 granulosa cells in BPS treated animals ($p<0.001$, Figure 11A). An ANOVA with Bonferoni post hoc test identified significant differences in Ki67 expression in primary 2 granulosa cells between the three EE_2 challenged treatment groups; the expression of Ki67 in the BPS treated group was significantly increased compared to the control ($p<0.001$) and TBBPA ($p<0.001$) treated groups (Figure 11A). In contrast, EE_2 induced a significant decrease in the level of Ki67 expression in secondary follicle theca cells and total theca cells in TBBPA treated animals (Figure 11B,C). Additionally, there were treatment related differences in ERα expression of EE_2 challenged animals for total percentage of ERα positive granulosa cells, although these did not reach statistical significance (ANOVA, $p=0.096$, Figure 11D). Pairwise T-tests did not identify the differences between treatment groups.

4.5.2 Week 16

There were no significant changes observed in Ki67 or ERα expression at 16 weeks of age for any of the follicle categories scored (data not shown).

4.6 Analysis of ovarian gene expression via qPCR

To further quantify changes in ovarian development influenced by early life chemical exposure, several genes which are crucial for hormone regulation in the ovary were measured based on their expression levels in ovarian tissue with the use of qPCR. Changes in ovarian gene expression levels were assessed for the following six target gene

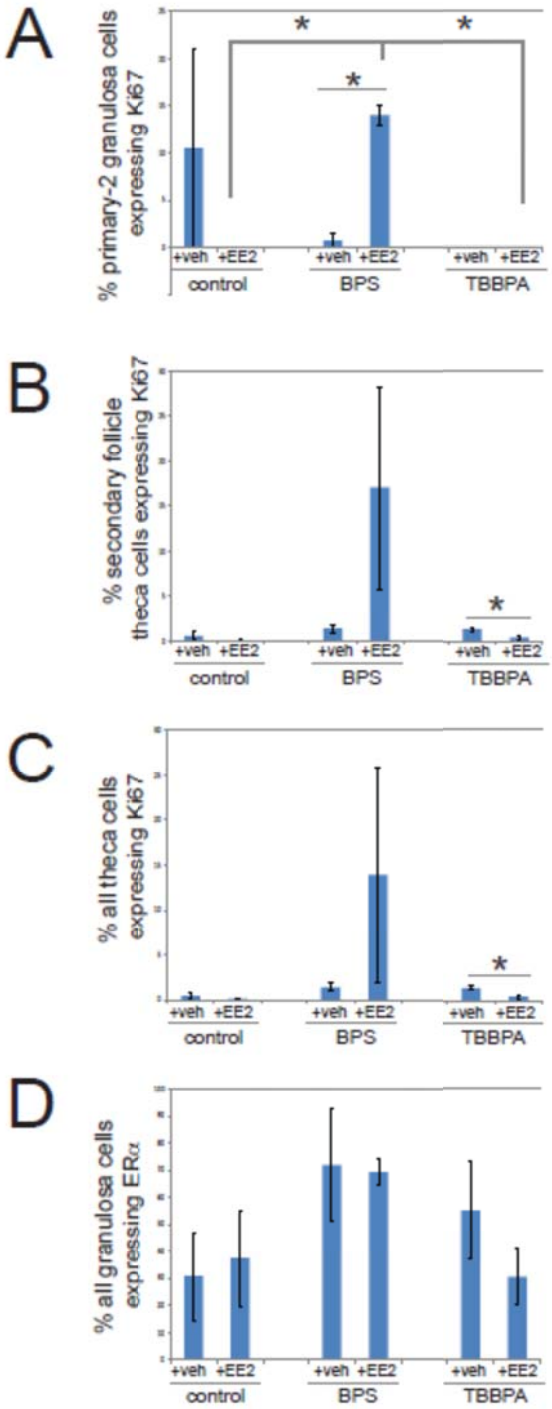


Figure 11. Quantification of expression of Ki67 and ERα from immunohistochemistries at PND22. Noteable effects were observed for A) expression of Ki67 in granulosa cells of primary-2 follicles; B) expression of Ki67 in theca cells of secondary follicles; C) expression of Ki67 in all theca cells; D) ERα expression in all granulosa cells. For all panels, * $p<0.05$, Bonferroni posthoc test.

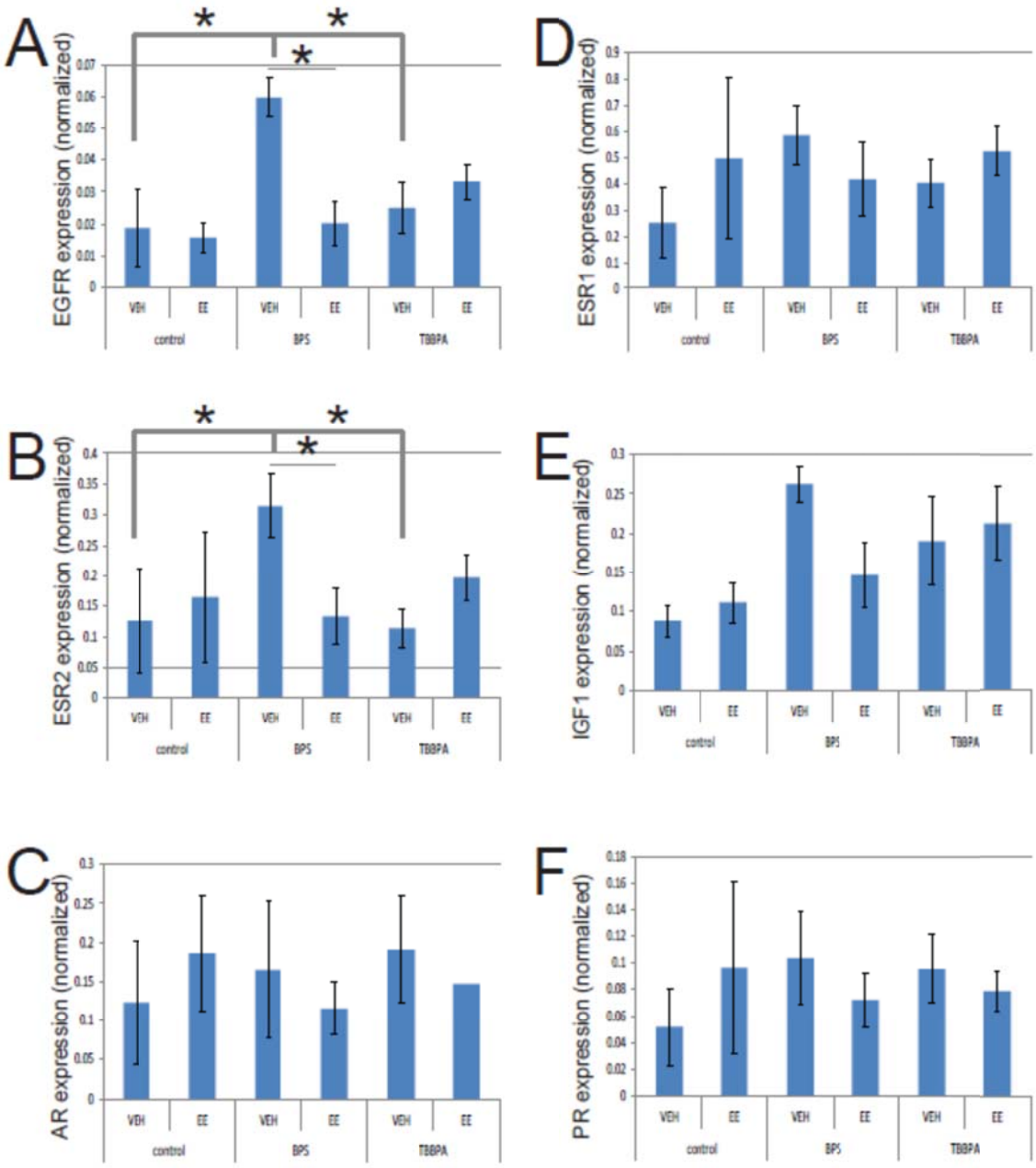


Figure 12. Summary of qPCR data from PND22 ovaries. A) EGFR, B) Esr2, C) AR, D) Esr1, E) IGF1, F) PR. * $p<0.05$, Bonferroni posthoc test.

sequences: androgen receptor (AR), epidermal growth factor receptor (EGFR), estrogen receptor α (ERα), estrogen receptor β (ERβ), insulin like growth factor1 (IGF1) and progesterone receptor (PR) (described in Section 2.8).

4.6.1 PND 22

EE_2 induced a significant decrease in EGFR and ERβ expression in animals exposed to BPS during prenatal development (p=0.003, Figure 12A, B). Significant changes in EGFR expression based on prenatal treatment were also noted; BPS treated animals exposed to vehicle at pre-puberty had significantly higher levels of EGFR expression compared to control animals (Bonferroni, p=0.023) and TBBPA treated animals (p= 0.034, Figure 12A) also exposed to vehicle. A significant increase in ERβ expression levels was also observed in BPS treated animals compared to TBBPA treated animals (p=0.05, Figure 12B). Significant differences based on treatment were not observed for AR (Figure 12C), ERα (Figure 12D), IGF1 (Figure 12E), or PR (Figure 12F).

4.6.2 Week 16

There were no significant or notable differences in ovarian gene expression at 16 weeks of age for any of the genes examined (Figure 13).

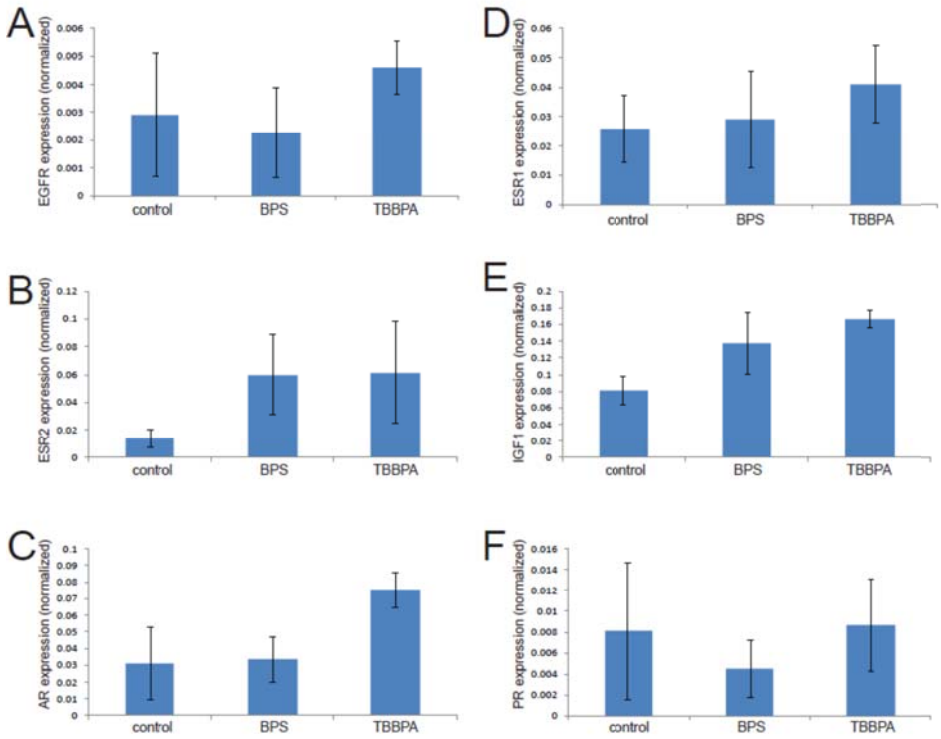


Figure 13. Summary of qPCR data from 16 week old ovaries. A) EGFR, B) Esr2, C) AR, D) Esr1, E) IGF1, F) PR.

CHAPTER 5

DISCUSSION

5.1 Body and uterine weight

Classic toxicological animal studies often assess risk associated with chemicals based on outcomes such as changes body weight, as this outcome is described in internationally recognized guideline studies (Catanese et al. 2015; Myers et al. 2009b) However, this measure is now viewed as an extreme health outcome since large doses of chemical are often needed to obtain a significant fluctuation in body weight. Here, significant changes in body weight of animals exposed to either BPS or TTBPA were not observed. Environmentally relevant, low-doses of chemical were administered in this study since BPS and TBBPA are suspected EDCs, and many modern EDC studies aim to examine doses below the toxicological no observed adverse effect level (NOAEL) (Vandenberg et al. 2012). Therefore, we did not expect to see changes in body weight as an outcome of chemical exposures since these classical signs of toxicity are only anticipated at higher doses.

To our knowledge, studies assessing changes in rodent body weight as a result of BPS exposure have not yet been conducted or reported. However, it has been shown previously that no significant changes in body weight were observed in rats exposed to TBBPA or BPA postnatally (Choi et al. 2011) or perinatally (Bo et al. 2010; Tada et al. 2006), although many other studies have shown increases in body weight and more sensitive measures of adiposity in rodents exposed to BPA during early development, reviewed in (Rubin and Soto 2009). Thus, assessments of the effects of EDCs on body

weight are complicated, and should consider whether weight gain and weight loss are both toxicologically relevant hazard assessment outcomes.

The uterus is particularly sensitive to estrogen since E_2 is responsible for stimulating proliferation of the endometrium and enlargement of the uterine glands during normal reproductive cyclicity (Kovacs and Ojeda 2011). Recommended tests issued by the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) of the U.S. Environmental Protection Agency (U.S. EPA) include what is known as the "uterotrophic response assay" as the preferred *in vivo* screening method for measuring estrogenicity and anti-estrogenicity of chemicals in rodents (Padilla-Banks et al. 2001). This method is a part of "tier 1" of a two tiered testing protocol meaning that a positive uterotrophic assay would require further testing as a part of "tier 2" (Zoeller et al. 2012). Briefly, this method compares wet weights of uteri from pre-pubertal female rodents exposed to a chemical of interest and compares the exposed uterine weights to the wet uterine weights from pre-pubertal females exposed to a known estrogenic compound such as EE_2 (positive control). If the chemical of interest induces a significant increase in uterine weight, similar to that induced by the positive control, that chemical may then be categorized as an estrogenic compound. In an alternative version of the uterotrophic assay, animals may be ovariectomized, then exposed to a chemical of interest; if the uterine weight increases significantly, the chemical may then be categorized as an estrogenic compound. In our study, we did not ovariectomize the animals, allowing us to preserve the ovaries for study, as uterine weight was not among the primary endpoints of interest.

Unfortunately, we did not weigh the uteri collected from females at PND22. To overcome this, another student in the laboratory is measuring additional endpoints that are predictive indicators of estrogen exposure such as height of the uterine endometrium, volume fraction of the uterine compartments, and expression of estrogen-sensitive proteins such as lactoferrin (Vandenberg et al. 2006). These data are forthcoming. At 16 weeks of age, we observed a significant change in uterine weight; animals exposed to BPS had significantly heavier uteri than animals exposed to TBBPA. However, we did not account for estrous cycle stage, which can drastically influence uterine weight. Thus, our results could simply be due to differences in the estrous cycle stage when animals were killed.

Further, because animal body weights varied, the relative uterine weight (uterine weight/body weight) was calculated for each animal to account for variability in body weight. No significant differences were observed for relative uterine weight for any treatment group. This could mean that the significant difference observed in absolute uterine weights between BPS and TBBPA treated animals may be attributed to differences in total body size, rather than specific effects of these compounds on uterine weight.

Previous studies show that prepubertal uteri are more sensitive to estrogen stimulation than uteri from adult animals (Kang et al. 2000; Padilla-Banks et al. 2001). Based on this information, it is possible that we did not observe significant changes in body/uterine weight ratios due to the age of the animals. It has been previously shown that TBBPA exposure during the perinatal period did not induce changes in uterine to body weight ratios at PND27 in rats (Tada et al. 2006). Although we cannot say whether

or not changes in uterine weights would be induced by perinatal BPS or TBBPA exposure when organs were collected at PND 22, it is likely that pre-pubertal EE_2 treatment would induce an increase in uterine weight. In fact, the timing of EE_2 treatment and the dose we utilized were both selected to induce a uterotrophic response; our major goal was to understand whether perinatal exposure to BPS or TBBPA would alter the estrogen-sensitive responses to this pubertal EE_2 challenge.

Although the EPA currently uses the uterotrophic assay to assess estrogenicity and anti-estrogenicity of chemicals, this method has stirred debate from endocrine and environmental health experts who suggest that the method is not sensitive enough to reliably measure estrogen inference caused by environmental chemicals (Zoeller et al. 2012). For example, chemicals with known endocrine disrupting properties at low doses including BPA and PCBs fail to induce uterotrophic response at the same low doses that induce other adverse health outcomes such as uterine tumors (Newbold et al. 2009; Rajapakse et al. 2002). The uterotrophic assay also fails to account for the increased sensitivity to estrogens after developmental exposures since fetal and neonatal exposures are not tested in this assay. Fetuses and neonates are more sensitive to environmental chemicals than adults, with effects often seen at doses hundreds or thousands of times lower than the doses required to observed uterotrophic responses in pubertal or adult ovariectomized animals (Markey et al. 2001), and see also discussion of the DOHaD hypothesis in section 1.5. It has been shown that fetal exposure to low doses of BPA results in adverse health effects later in life including preneoplastic mammary lesions that are not observed at high doses (Durando et al. 2007; Murray et al. 2007). Effects due to developmental and low dose chemical exposures are completely overlooked by

regulatory measures of estrogenicity by the endocrine disrupter screening program (EDSP). For these reasons, endocrinologists view the uterotrophic assay as unfit to optimally measure estrogenic effects induced by environmental chemicals (Zoeller et al. 2012).

5.2 Behavioral effects induced by perinatal chemical exposure

It has been shown previously that perinatal exposure to estrogenic chemicals such as BPA can induce alterations in the number of neurons and expression of genes in sexually dimorphic regions of the brain and thus lead to changes in sexually dimorphic behaviors in assays including the open field test (Patisaul et al. 2006; Rubin et al. 2006). Behavior data from open field tests in rodents suggest that females have a higher rate of activity in comparison to males (Archer 1975; Blizard et al. 1975). Classical endocrinology studies revealed that the aromatization of testosterone to estrogen during fetal development is required for the development of male brain phenotypes in the male rodent; thus, exposures of estrogens to females during these critical periods of development can induce masculinization of these brain regions, and thus masculinization of behaviors observed in the open field (Ogawa et al. 1997; Simerly 1989). A previous study reported sexually dimorphic behaviors such as rears on wall, time spent in the center of the field, and time stopped, in control animals; however, in animals that were developmentally exposed to environmentally relevant doses of BPA, these sexually dimorphic behaviors vanished with similar behaviors observed in males and females (Rubin et al. 2006). This loss of sexual dimorphism appeared to be due to masculinization of female behaviors, and feminization of male behaviors.

Although our experiments did not compare behaviors between male and female mice, our goal using the open field test was to identify potential differences in behavior induced by developmental chemical exposure that could be indicative of alterations to sexually dimorphic areas of the brain. The parameters measured in the open field test were specifically chosen to measure activity level of the animals, with the expectation that females exposed to exogenous estrogenic substances during development would display decreased activity in the open field test, indicating masculinization due to exposure.

In contrary to the previously mentioned BPA study (Rubin et al. 2006), the behavioral data reported here do not suggest that females developmentally exposed to BPS exhibit masculinized behaviors in comparison to control females at 15 weeks of age. Instead, four parameters (center rears, wall rears, center crosses, total rears) suggest that animals exposed to BPS have increased activity in comparison to the control, consistent with hyperactivity. There is no evidence that we are aware of that behaviors in the open field can be "hyper-feminized", although that conclusion is also consistent with our observations.

In contrast to the effects of BPS, females exposed to TBBPA had decreased activity compared to the control. These findings could suggest that animals exposed to TBBPA are masculinized to some degree, although additional behavioral measurements, as well as investigations of sexually dimorphic regions of the brain, are also needed to support such a conclusion.

Importantly, significant behavioral differences were not seen at 23 weeks of age, although a notable decrease in the number of center crosses induced by TBBPA exposure

compared to control animals could further support the hypothesis that developmental exposure to TBBPA results in masculinization to some degree. It is plausible that the behaviors observed in the open field were affected by the estrous cycle stage of the females, which was not controlled in our study at either 15 or 23 weeks of age. Again, additional study is needed here.

5.3 Effects on ovarian follicle formation, protein expression, and gene expression in prepubertal animals (PND 22)

It has previously been reported that developmental exposure to estrogenic EDCs such as BPA and DES result in abnormal ovarian follicle formation (Hunt et al. 2012; Li et al. 2014; Rivera et al. 2011; Rodriguez et al. 2010; Wang et al. 2014). Based on the hypothesis that BPS and TBBPA induce estrogenic effects and thus induce premature ovarian development, we compared morphology of ovarian follicles at PND 22 from animals developmentally exposed to BPS and TBBPA with ovarian follicles from females exposed to oil only. Importantly, a critical part of our experimental design included a pre-pubertal estrogen challenge. One female from each litter was treated with oil and one was treated with 1 μg/kg/day EE_2 for three days, from PND19-PND21. Prior studies have revealed that developmental exposures to EDCs including BPA alter the response of animals to hormonal or carcinogen challenges experienced later in life (Durando et al. 2007; Prins et al. 2007; Wadia et al. 2007).

Pairwise T-tests of follicle formation data revealed that EE_2 induced significant follicular growth in TBBPA exposed and control animals at PND22 in the following categories: total number oocytes, total secondary follicles and total number of follicles. Follicular growth and/or development here is defined as the transition of primordial

follicles to any other type of follicle with increased maturity in regards to the follicle cycle (described in 1.4.1). We would expect to see an increase in follicular development induced by EE_2 since increased estrogen levels play a crucial role in the onset of puberty. However, this induction of growth was not observed for animals exposed perinatally to BPS and challenged with EE_2. Based on our results, developmental exposure to BPS alone induced follicular growth (observed in females at PND22 after pre-pubertal treatment with oil vehicle); this was in contrast to control and TBBPA treated animals, which required EE_2 to induce follicular growth in the pre-pubertal period. Collectively, these results suggest that perinatal exposures to BPS induce premature development of the pre-pubertal ovary, and that additional estrogen exposures have no additive effect, further suggesting that developmental BPS exposures disrupt normal estrogen regulated pathways.

Further statistical analyses revealed that the number of oocytes in four sections of ovary was significantly lower in BPS treated animals compared to control animals after an EE_2 challenge. These data also support our conclusion that a pre-pubertal estrogen challenge provided by EE_2 was not successful at increasing oocyte development in BPS treated animals. Since the dose of EE_2 we selected simulates estrogen levels experienced during and after puberty, these data may suggest that the number of oocytes formed after puberty could be significantly reduced in BPS-treated animals; these results may indicate that BPS could pose a threat to the individual's fertility since a decrease in the number of oocytes directly correlates to decreased chances of fertilization.

Several published studies have reported significant changes in the number of specific types of ovarian follicles induced by developmental exposure to BPA in mice,

rats and lambs (Li et al. 2014; Rivera et al. 2011; Rodriguez et al. 2010; Wang et al. 2014). All of the studies above observed a significant decrease in the number of primordial follicles after BPA exposure, which could be consistent with our results suggesting that BPS causes an increase in ovarian maturation; increased ovarian maturation would be expected to decrease the number of primordial follicles, although we did not observe this specific measure. In contrast to our findings, one study observed reduced numbers of primary, antral and total follicles in animals exposed to BPA prepubertally (Li et al. 2014). To our knowledge, no studies using developmental EDC exposures and a pre-pubertal estrogen challenge have been conducted using ovarian endpoints, making it difficult to make further comparisons with our study.

Although these results were not statistically significant, BPS-treated females had an increase in the numbers of secondary follicles compared to controls. Secondary follicles are a measure of follicular maturity; because follicular maturation does not normally occur until the onset of puberty, it may be concluded that BPS exposure alone can advance follicular maturity. This data is similar to that of others who reported increases in the number of growing follicles (primary and preantral) induced by developmental BPA and DES exposure, consistent with the hypothesis that early life exposures to estrogenic chemicals can stimulate the transition of primordial follicles to more mature follicles (Rivera et al. 2011; Rodriguez et al. 2010).

Although time of puberty was not specifically recorded in these experiments, the increase in follicle maturation suggests that puberty and ovulation in animals exposed to BPS could possibly have been reached sooner than in control and TBBPA treated animals. It has been reported that developmental exposure to BPA induces early onset of

puberty in rodents (Nah et al. 2011; Nikaido et al. 2005; Wang et al. 2014). Increased urinary BPA concentrations have also been associated with early onset of puberty in humans (Caserta et al. 2014).

To further test the hypothesis that BPS and TBBPA are able to induce estrogenic signaling pathways that prematurely induce ovarian development, we compared gene and/or protein expression of factors that tightly regulate hormone action in the ovary at PND 22. Immunohistochemistry was used to measure the expression of Ki67, a marker of cell proliferation. EE_2 treatment induced increases in the number of Ki67 positive granulosa cells in specific follicle types in the ovaries of BPS exposed animals. Further, these increases were significant when BPS treated animals were compared to either the control or TBBPA treated groups. These data were surprising because they do not appear to agree with the follicular data discussed above. Based on the follicle data, which showed that EE2 induced follicular development in the control and TBBPA but not BPS groups, we might expect to see no effect on measures of cell proliferation in animals exposed to BPS and challenged with EE_2. Instead, our results suggest the possibility that the premature induction of ovarian development by BPS is independent of proliferation, or the more likely possibility that BPS induced a wave of proliferation prior to our assessment at PND22, and a second wave of proliferation is induced by the EE_2 challenge in these BPS-exposed females. Our immunohistochemical proliferation results are somewhat consistent with previous studies that observed increased expression of Ki67 in granulosa cells of growing follicles after developmental exposures to BPA or DES (Rivera et al. 2011; Rodriguez et al. 2010).

In addition to our analysis of proliferation in granulosa cells, our immunohistochemical analysis revealed fewer Ki67 positive theca cells in secondary follicles and in the overall percentage of positive theca cells in TBBPA treated animals after an EE_2 challenge. These results are contrary to a previous study that reported an increase in Ki67 expression in antral thecal cells as a result of BPA exposure. Our data suggest that, at least for some measures in the ovary, TBBPA exposures induce different effects than either BPA or BPS; our data, as well as results from a number of prior studies, are consistent with the hypothesis that both BPS and BPA act similar to estrogen. Thus, our data suggest that developmental exposures to TBBPA alters ovarian development via mechanisms that are distinct, and perhaps even independent of estrogen receptor signaling.

Both immunohostochemistry and qPCR analyses were used to quantify the expression of ERα, one of the two main types of nuclear receptor activated by estrogen to mediate estrogenic effects in ovarian follicles (Peretz et al. 2012). Although qPCR is widely accepted as a reliable measure to quantify gene expression, immunohistochemistry was performed to allow visualization of potential changes in ERα expression in specific types of cells and follicles. Our analyses revealed that EE_2 induced an increase in the percentage of ERα positive granulosa cells in animals exposed to BPS, although this increase was not statistically significant. Further, ovaries from animals exposed to BPS with and without an EE_2 challenge showed a trend of increased ERα expression in many follicle categories compared to control and TBBPA treated groups. However, no significant changes in ERα expression were observed with qPCR. Previous studies examining ERα expression via immunohistochemistry and qPCR after BPA

exposures have shown similar mixed findings; some studies demonstrate increased ERα expression after BPA exposure (Rodriguez et al. 2010) while others did not observe any changes in ERα expression induced by BPA (Rivera et al. 2011; Veiga-Lopez et al. 2013). Yet, these prior studies may shed light on how an estrogenic compound can influence ERα protein expression, measured by immunohistochemistry, and not influence expression of the ERα gene, Esr1. *In-vitro* timecourse experiments have shown that the effects of BPA on ERα expression are time dependent; these studies have also concluded that BPA does not mediate follicular growth through ERα, but via disruptions to other aspects of the cell cycle (Peretz et al. 2012). In fact, ERα itself has been shown to play little or no role in follicular development parameters (Hegele-Hartung et al. 2004).

The other main nuclear receptor that regulates estrogenic effects in ovarian follicles and drives granulosa cell proliferation is ERβ (Peretz et al. 2012). We observed that perinatal exposure to BPS increased ERβ expression (Esr2) in the whole ovary compared to control and TBBPA treated animals. Yet, EE2 challenge caused a decrease in Esr2 expression in BPS-treated females. This data may help explain the results we observed for the effects of BPS on follicular data discussed above, where BPS exposure increased the number of advanced follicles. An increase in ERβ expression is consistent with an increase in follicle growth (Peretz et al. 2012). Our findings also agree with prior studies which revealed an increase in ovarian ERβ expression as a result of BPA exposure (Brieno-Enriquez et al. 2012; Peretz et al. 2012; Rodriguez et al. 2010). Some previous studies have concluded that BPA acts as an ERβ antagonist, and via these actions can disturb ovarian follicular development (Brieno-Enriquez et al. 2012; Hunt et al. 2003; Susiarjo et al. 2007). In fact, it has been noted that ovarian meiotic defects

induced by developmental BPA exposure are nearly identical to those observed in ERβ knock-out mice, highlighting the crucial role of ERβ function in ovarian development (Susiarjo et al. 2007). Our findings mirror those induced by BPA, suggesting that BPS also acts as an estrogen antagonist and may be able to bind to ERβ, thus inducing downstream changes which result in the disturbance of ovarian follicular development. Finally, our findings agree with the hypothesis that ERβ mediates direct estrogen effects on ovarian follicular development (Hegele-Hartung et al. 2004). The antagonistic actions of BPS on ERβ could inhibit endogenous estrogen activation of ERβ, thereby disrupting negative feedback; ovarian cells may compensate for the loss of ERβ function by increasing ERβ expression, consistent with the increased Esr2 expression shown here.

Similar to the ERβ expression results discussed above, BPS exposure also increased expression levels of epidermal growth factor receptor (EGFR) compared to control animals. It has been shown that EGFR expression increases late in folliculogenesis, consistent with our finding of increased numbers of mature follicles in BPS treated animals (El-Hayek et al. 2014). The increase in EGFR expression due to BPS exposure may have serious implications. Increased expression of EGFR is a characteristic of ovarian carcinoma; EGFR over expression is present in 25-50% of all ovarian cancers (Lafky et al. 2008). Our data suggests that BPS may have malignant effects in regard to ovarian development and poses a public health concern. Yet, importantly, treatment with an EE2 challenge decreased EGFR expression in BPS-treated animals.

EGFR expression is critically regulated by E_2; both estrogen and FSH stimulate EGFR expression in granulosa cells, and the LH surge that is responsible for ovulation releases ligands that activate EGFR in granulosa cells (El-Hayek et al. 2014; Garnett et

al. 2002). These factors suggest several plausible phenomena: First, BPS may be able to act as an estrogen to stimulate increased EGFR expression in granulosa cells. Alternatively, BPS may be able to induce an LH surge in BPS treated animals, which would further explain the increase in EGFR expression. The observed effects induced by BPS exposure including increased EGFR and ERβ expression, in combination with data showing increased follicular maturation, go hand in hand; follicular growth is dependent on estrogen activation of ERβ and puberty induces follicular growth and increased EGFR expression in granulosa cells.

5.4 Effects on follicle formation, protein expression, and gene expression in adult animals (16 weeks)

In order to capture effects that develop later in life after developmental chemical exposure, the same criteria that were evaluated at PND 22 were also tested at 16 weeks of age. In contrast to the many effects observed in the ovary at PND22, few effects of BPS or TBBPA were observed in adulthood. At 16 weeks of age, no effects were observed on gene expression, cell proliferation, or follicle stages. Slight effects may exist for frequency of ovarian cysts, which was raised in BPS-treated animals compared to controls at this age.

Previous studies that have examined developmental EDC exposure and related ovarian outcomes have largely failed to examine ovarian endpoints later in life. Instead, these studies have focused on ovarian development in fetal and/or prepubertal animals. This lack of historical information makes it difficult to interpret the data found here.

Epidemiological studies focusing on precocious puberty induced by developmental exogenous estrogen exposures report varied negative health effects once

these affected individuals reach adulthood (reviewed in (Unuvar and Buyukgebiz 2012)). It is plausible that the adult age assessed here (16 weeks) was too young to see subtle effects on the reproductive system. We plan to assess tissue from 24-week-old animals from the same cohort in order to determine whether the significant changes observed at PND 22 correlate with additional changes at a time point even later in life. On the other hand, several rodent and epidemiological studies have found an association between EDC exposure and impaired fertility (Peretz et al. 2014; Souter et al. 2013). To our knowledge, to the effects of BPS or TBBPA on fertility or fecundity has not yet been explored; additional studies are needed to confirm actual effects on fecundity, such as reduced litter size, reduced numbers of litters, and altered time to achieve pregnancy (Cabaton et al. 2011) .

The contrast in effects seen at PND 22 and week 16 may also be due to differences in circulating hormone levels before and after puberty. At PND 22, mice typically have not yet entered the pubertal window of development. During this window, sex steroid hormones are low and start to increase in preparation for the onset of puberty. With the very low production of estradiol prior to puberty (typically onset after PND25 in female mice), we can conclude that the effects seen at PND 22 were induced by an exogenous factor that has estrogenic function since the measured endpoints of interest were estrogen dependent. Yet, at the onset of puberty, the ovary produces estradiol, which initiates hormonal positive and negative feedback systems involving the ovary. It is plausible that significant changes were not observed at 16 weeks of age because endogenous estrogen levels were high enough to block the effects of early life BPS

exposures. Control and TBBPA treated animals at 16 weeks of age would be "caught up" in terms of sexual maturity and thus indistinguishable from animals treated with BPS.

5.5 Future directions

Future experiments will include analysis of the outcomes measured above for animals of the same cohort at week 24 of age, focusing on follicular and gene expression changes. Measures of ovarian aging will also be assessed with the use of TUNEL assay for animals in each age group to quantify the number of apoptotic follicles induced by treatment and age. Developmental BPA exposure has been shown to increase the number of atretic follicles of the ovary (Li et al. 2014; Peretz et al. 2012; Rivera et al. 2011; Wang et al. 2014). Additional measures of gene expression for genes indicative of normal folliculargenesis including cyclin-dependent kinase (Cdk) inhibitor 1B (p27), and genes responsible for ovarian development including Cyp19, the enzyme responsible for the conversion of androgens into estrogens, and 5α-reductase, the enzyme responsible for the conversion of testosterone into dihydrotestosterone, could be analyzed. Expression of these genes in the ovary has been altered by developmental BPA exposure (Rivera et al. 2011; Rodriguez et al. 2010; Veiga-Lopez et al. 2013)

As mentioned above, in the long-term, determining whether BPS and TBBPA alter measures of fertility and fecundity remains an important future research need. Not only do these measures represent obvious adverse outcomes, they suggest possible endpoints with public health implications. Further, analyses that examine additional aspects of the HPO axis including gene expression in the hypothalamus and pituitary will

also help to elucidate the mechanisms by which these compounds alter ovarian development and responsiveness.

5.6 Conclusions

At the start of these experiments, it was not yet clear whether BPS or TBBPA should be considered endocrine disrupting chemicals. In the last year, a number of studies on BPS have suggested that developmental exposures to this compound induce altered health outcomes in rodents and zebrafish (Rochester and Bolden 2015). The results acquired through the experiments described here are fairly consistent with my initial hypotheses that developmental exposure to BPS and TBBPA 1) induce changes in estrogen-sensitive endpoints 2) disrupt cellular and molecular events in the developing ovary and 3) act as estrogens.

My results are consistent with hypothesis 1; BPS and TBBPA are able to induce changes in the ovary, an estrogen dependent endpoint. Hypothesis 1 is directly related to hypothesis 2 in that, changes in ovarian development induced by BPS and TBBPA were observed on both a cellular and molecular level. Majority of the cellular and molecular effects observed here were a result of BPS exposure. At the cellular level, these changes were most pronounced through the responsiveness of the ovary to a prepubertal estrogen challenge, which revealed that BPS induces premature ovarian development and alters the responsiveness of the ovary to EE_2. At a molecular level, the estrogen challenge of animals exposed to BPS revealed that these animals are still responsive to estrogens and that this responsiveness may be mediated through ERβ, confirmed by qPCR data

showing elevated ERβ expression. The ability of BPS to act via ERβ confirms my final hypothesis of this chemical's ability to act as an estrogen and therefore I would classify BPS as an EDC. TBBPA on the other hand appears to act through a different mechanism than BPS. Surprisingly, my study revealed that TBBPA also alters ovarian development on the molecular level; however, these effects may be independent of actions via the estrogen receptor. Majority of the molecular effects induced by TBBPA were observed in theca cells that primarily produce androstenedione for granulosa cells to convert to estradiol via aromatase. It is possible that TBBPA disrupts the production of androgens and thus the synthesis of estradiol without directly binding to estrogen receptors. This proposed mechanism of endocrine disruption induced by TBBPA is different than that of BPS however; endocrine function is modified in both cases.

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